Hematologic & Immunologic Diseases I

1. SPK-9001: Adeno-Associated Virus Mediated Gene Transfer for Hemophilia B - Sustained Fix Activity, Persistent Endogenous Prophylaxis and Improved Quality of Life at One Year and Beyond

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Background: Prior work achieved long term expression of factor IX (mean FIX:C ~5.1%) following AAV8-mediated gene transfer in hemophilia B (Nathwani *et al.,* 2014). Transgene-derived FIX:C fell short of the ~12% level that may be necessary to eliminate spontaneous hemarthroses (den Uijl *et al.* 2011). We developed a vector capsid and expression cassette that could, at low doses, achieve FIX:C adequate to prevent bleeding without use of exogenous FIX. We report 1 year follow up data in 10 subjects following a SPK-9001 dose of 5 x 10^{11} vg/kg. **Objective:** Consistent, predictable and persistent trans-gene-derived FIX:C >12% adequate to prevent spontaneous bleeding without prophylactic FIX infusions. **Methods**: SPK-9001 combines a liver specific, bioengineered AAV capsid (Spark100) and a single-stranded codon optimized expression cassette encoding FIX-Padua. FIX-Padua confers ~8-12-fold greater specific activity compared to wild-type FIX (Simioni *et al.*2009, Crudele *et al.* 2015). Subject baseline characteristics were determined at study screening. FIX:C and clinical data, prospectively collected after vector infusion, were compared to pre-infusion data. **Results**: As of 1/15/18, 12 subjects were dosed with SPK-9001 (5x10^{11} vg/kg) and 10 had completed 1 year follow up. Subjects are males ages 18-61 with FIX:C ≤2%. No vector related serious adverse events (SAEs) or FIX inhibitors occurred. Mean FIX:C at 12 weeks following vector (30.0±17.6%) did not significantly differ from FIX:C at 52 weeks (35±21%). Three subjects have FIX:C persistently > 50%. One subject had pre-treatment Spark100 neutralizing antibody (NAb) titer of 1:1 and the remaining were <1:1. FIX:C for subjects with <1:1 NAb was 35±13% at 52 weeks. FIX:C in the five subjects with a history of HCV and stage 1-2 liver fibrosis or with combined HIV and HCV exposure did not differ significantly from other participants. All subjects discontinued prophylaxis. Use of factor concentrate occurred in 2 subjects one for target joint bleeding and one for a thigh bleed treated shortly after vector infusion. Two subjects were treated with a steroid taper for suspected T-cell mediated capsid immune responses. Baseline characteristics did not differ from other subjects in the cohort. Both subjects have stable, sustained FIX:C (14% and 78%) with no bleeding post vector. The first subject dosed is greater than two years post infusion with no decline in FIX activity since reaching his stable level which occurred at about 12 weeks. In the 10 subjects who are 52 weeks post infusion, significant mean improvements from baseline to post vector were found for the Haem-A-QoL domains 'Future' ($p<0.005$), 'Treatment' ($p<0.008$), and 'Total Score' ($p<0.001$), and fifty percent of the patients were more physically active post vector. **Conclusion**: These data represent the highest, most consistent and sustained vector derived FIX:C following gene transfer in the largest cohort of HB subjects treated using the same vector and dose with 1 year follow up. All subjects achieved improved QoL, termination of prophylaxis, near complete elimination of bleeding and factor use without SAEs. Low grade liver fibrosis and prior HCV exposure did not appear to affect achieved FIX:C.

2. Lentiviral Hematopoietic Stem Cells Gene Therapy for Beta-Thalassemia: Update from the Phase I/II TIGET BTHAL Trial

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Beta-thalassemia is a genetic disorder due to mutations in gene encoding the beta-globin chain causing a reduced or absent production of hemoglobin A. The only curative treatment is represented by allogeneic bone marrow transplantation but still available for a minority of patients and associated with risk of complications and mortality. In 2015, we started a phase I/II gene therapy clinical trial (NCT02453477) based on the autologous transplantation of G-CSF and plerixafor mobilized hematopoietic stem cells engineered by lentiviral vectors expressing a transcriptionally regulated human beta-globin gene and a myeloablative treosulfan and thiotepa conditioning regimen favoring efficient engraftment with reduced toxicity. The chosen route of administration of gene modified cells is intraosseous with the aim of enhancing engraftment and minimizing first-pass intravenous filter. The clinical study foresees treatment of 10 patients: 3 adults followed

by 7 minors, with a staggered enrolment strategy based on evaluation of safety and preliminary efficacy in adult patients before inclusion of pediatric subjects. Up to now, nine patients with different genotype have been treated with a mean cells dose of $19.0x10^6 \pm 1.1x10^6$ cells/kg containing an average 59.4±10.7% of transduced progenitors cells and VCN of 0.93±0.27. The procedure was well tolerated, with no product-related adverse events. Preliminary results related to patients who achieved a 1 year follow-up, showed a robust and stable engraftment of gene modified cells in 6 out of 7 patients. At latest FU, transduced BM progenitors ranged between 12.6 and 76.4%, and vector-transduced cells were detected in multiple lineages. Polyclonal vector integrations profiles have the expected genomic distribution and no evidence of clonal dominance. So far, the clinical outcome indicates reduction in transfusion requirement in adult patients and greater clinical benefit in younger subjects with 4 out of 5 patients who discontinued transfusion shortly after gene therapy. Prolonged follow up analysis will provide additional information on the long-term safety and clinical efficacy of this treatment.

3. Gene Editing of FOXP3 in Primary CD4+ T Cells to Generate Antigen-Specific Engineered T_{reg} Products

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$CD4^+$ regulatory T cells (T_{reg}) are immunosuppressive lymphocytes with critical roles in the maintenance of immune tolerance. The generation of immunotherapies to augment T_{reg} function in transplant recipients or patients with autoimmune disorders is therefore of great interest. With this goal, clinical trials for treating graft vs. host disease (GvHD) and Type I diabetes (T1D) by re-infusion of autologous natural T_{reg} (nT_{reg}) cells that have been expanded *ex vivo* have been conducted. While these trials indicate this strategy to be safe and potentially therapeutic, they also highlight limitations to this approach, including the slow *ex vivo* expansion and potential instability of nT_{reg} *in vivo*, as well as cell intrinsic T_{reg} defects inherent to some autoimmune diseases. We previously developed a gene editing strategy using TAL effector nucleases combined with AAV6 donor template delivery to drive expression of Forkhead box P3 (FOXP3), the key transcription factor orchestrating T_{reg} development and maintenance. We found that delivery of a strong promoter upstream of the *FOXP3* coding sequence of peripheral blood $CD4^+$ cells overcame epigenetic transcriptional repression, driving high levels of FOXP3. In turn, the transcriptome, phenotype, and function of these cells became re-programmed, exhibiting nT_{reg}-like properties. We thus termed these cells edited T_{reg} (edT_{reg}). Similar to nT_{reg}, polyclonal edT_{reg} delayed GvHD in NOD-*scid*-$Il2rg^{NULL}$ (NSG) mice after effector T cell (T_{eff}) co-infusion compared to T_{eff} infusion alone, or mock-edited T cell co-infusion. While polyclonal edT_{reg} are desirable for diseases such as GvHD where the autoantigens are many or unknown, antigen-specific edT_{reg} may improve the potency of this cell therapy with known autoantigens, such as T1D. To test the feasibility of generating antigen-specific edT_{reg}, in the current study we apply our genome editing strategy to both human and murine antigen-specific $CD4^+$ T cells and perform functional tests of immunosuppression both *in vivo* and *in vitro*. Using the same editing strategy developed for human cells, but using mouse-specific nucleases and donor templates, we edited myelin oligodendrocyte glycoprotein peptide (MOG_{35-55})-specific TCR transgenic (TCR^{MOG} or 2D2) murine T cells, generating MOG_{35-55}-specific edT_{reg}. These or polyclonal edT_{reg} were co-transferred with 2D2 T_{eff} cells into $RAG^{-/-}$ mice in an adoptive transfer model of experimental autoimmune encephalomyelitis (EAE; a murine model of multiple sclerosis). Here, we found that the antigen-specific edT_{reg} greatly suppressed T_{eff} expansion after MOG immunization, in comparison to polyclonal murine edT_{reg}. We next applied our gene editing strategy to flu and tetanus peptide-specific primary human $CD4^+$ T cells. Flu/tetanus-peptide-specific edT_{reg} displayed phenotypic similarity to nT_{reg} ($FOXP3^{high}$, $CD25^{high}$, $CD127^{low}$, $CTLA4^{high}$), and suppressed production of cytokines (IL-2, IL-17, and IFN-γ) compared to mock-edited antigen-specific and polyclonal controls. The immunosuppressive property of peptide-specific edT_{reg} was validated in a thymidine-based *in vitro* suppression assay. As an ongoing effort, we are currently developing approaches to generate islet-specific edT_{reg} to test *in vitro* and *in vivo* function. Taken together, our findings demonstrate successful generation of multiple antigen-specific edT_{reg} cell products with suppressive function *in vitro* and *in vivo*.

4. The Potential Use of Nanobodies Delivered *Via* AAV Vectors in the Treatment of Haemophilia

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The coagulation cascade is a finely-regulated process involving pro-coagulant factors (like factor VIII or factor IX) and anti-coagulants (like Antithrombin). The lack of factor VIII (FVIII) or factor IX (FIX) in hemophilia A (HA) and B (HB), respectively, shifts the balance to an inefficient blood clotting. In recent years, several promising gene transfer approaches for HA and HB treatment were tested. Among them, adeno-associated (AAV) vector mediated gene transfer demonstrated therapeutic efficiency and long-lasting expression of the missing factor. However, there are still some concerns related to the potential vector and/or transgene immunogenicity, especially for HA patients which often develop inhibitors against the replaced protein. We propose here an innovative AAV-based gene therapy approach for haemophilia care using single domain antibody fragments (nanobodies) derived from llama. These nanobodies target the antithrombin (AT) anticoagulant to re-balance the hemostasis in a FVIII- or FIX-independent manner. Nanobodies present several advantages, including a low immunogenic potential and limited need for humanization. Moreover, their small size (±17 kDa) facilitates their bio-engineering and the incorporation of their cDNA into viral vectors. In a first series of llama immunization and a single phage display procedure, several anti-AT nanobody sequences were isolated. Three

nanobodies were selected showing cross-reactivity with the murine AT and subsequently engineered to produce bi-valent and multi-valent variants. *In vitro*, their inhibitory activity was tested in thrombin generation tests using FVIII-deficient plasma. We showed that the bi-valent KB-AT-02/03 and the tri-valent KB-AT-113 nanobodies were able to correct thrombin generation to the same extent as FVIII. This effect was further confirmed by injecting the purified nanobodies into HA mice, in a tail vein transection model. While vehicle-injected mice showed consistent blood loss during the observation time (>750µl on average), mice injected with the nanobodies showed a strongly reduced blood loss (<300ul on average). We developed AAV8 vectors encoding for KB-AT-02/03 (AAV8-AT 02/03) and KB-AT-113 (AAV8-AT-113) under the control of a hepatocyte-specific promoter, in order to perform a tolerogenic gene transfer and express the nanobodies from the liver. In a preliminary *in vivo* test, AAV8-AT-02/03 vector was injected in HA mice, where we observed an increased AT-inhibition in plasma over time (AT activity was reduced up to 0.7% at 8 weeks post-injection). In a different model, both the AAV8-AT-02/03 and AAV8-AT-113 vectors were injected into HB mice and their efficacy was tested one month after administration by performing a tail clip bleeding assay. We observed a significant reduction in blood loss in animals injected with the AAV8-AT-02/03 (<300ul on average) and 113 (<600ul on average) compared to the vehicle-treated group (>800µl on average). We did not observe anti-nanobody immune-response in the treated animals. We demonstrated that nanobodies represent an innovative tool to effectively target antithrombin and re-balance the hemostatic process. In our setting they showed pro-coagulant activity, both *in vitro* and *in vivo*. Importantly, this gene therapy approach provides the potential of a long-term therapeutic solution in both haemophilia A and B patients with or without inhibitory antibodies to the therapeutic clotting factor.

5. Reconstitution of Hematopoiesis in Patients Treated with Gene Therapy for Beta-Thalassemia

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Beta-thalassemia (Bthal) is a genetic disorder due to mutations in the ß-globin gene, leading to a reduced or absent production of HbA, which interferes with erythroid cell maturation and limits red cell production. Patients are affected by severe anemia, hepatosplenomegaly, and skeletal abnormalities due to rapid expansion of the erythroid compartment in bone marrow (BM) caused by ineffective erythropoiesis. In a classical view of hematopoiesis, the blood cell lineages arise via a hierarchical scheme starting with multipotent stem cells that become increasingly restricted in their differentiation potential through oligopotent and then unipotent progenitors. Recently, it has been proposed a re-defined model of hematopoietic hierarchy in which in the adult BM, only two progenitor classes predominate. A general perturbed and stress condition is present in the thalassemic BM microenvironment and its impact on the hematopoietic cell subpopulations is mostly unknown. To address which model of hematopoiesis/erythropoiesis occurs in Bthal, we define by immunophenotype analysis the lineage commitment in patients affected by the pathology compared to healthy donors. Furthermore, in patients enrolled and treated in a phase I/II gene therapy protocol (TIGET BTHAL, ClinicalTrials.gov Identifier #NCT02453477) this type of analysis will allow to study the shape of reconstituted hemato/erythropoiesis by gene-modified transplanted $CD34^+$ cells. Differences in the primitive compartment were observed with an increased proportion of multipotent progenitors in Bthal patients compared to healthy donors. We were also able to unveil age-related differences, thanks to the availability of adult and pediatric subjects' samples. The analysis of $CD34^+$ cell subpopulations revealed changes in the composition of HSPCs during follow up. By subjecting the classically defined progenitor subsets to a new sorting scheme that efficiently resolved My, Ery, and Mk lineage fates, we quantified the new myeloid (My) and eryhroid (Ery) subsets within the $CD34^+CD38^+$ compartment and found a reduction of Ery subsets in Bthal samples. Moreover, to monitor the terminal erythroid differentiation, we analyzed the frequency of erythroid cells in distinct stages of differentiation by using specific markers, as GpA/Band3/integrin alpha4. In all analyzed pre-gene therapy cases, the proportion of erythroblasts at successive stages of differentiation did not follow the predicated doubling pattern that is observed in normal BM. This alteration in erythroid maturation steps was ameliorated in those treated subjects showing increased Hb level. Further studies on lineage commitment, exploiting LV marking for the *in vivo* tracking of single cell fate, will address which model of hematopoiesis/erythropoiesis occurs in Bthal and the impact of gene therapy.

6. Hematopoietic Stem Cell Gene Editing for the Treatment of Wiskott-Aldrich Syndrome

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Wiskott-Aldrich syndrome (WAS) is an X-linked primary immunodeficiency caused by mutations in the WAS gene and characterized by severe platelet abnormalities, defective cellular and humoral immunity and development of autoimmune diseases and cancer. Although gene therapy approaches using integrating lentiviral vectors showed encouraging results, these carriers bear the potential risk of genotoxicity and non-physiological transgene expression in target cells. It is therefore desirable to develop new strategies for targeted gene correction. The goal of our study is to use CRISPR/Cas9-based genome editing to knock-in a wild-type WAS cDNA in its first coding exon, allowing transcriptional regulation from WAS endogenous promoter and functional correction of the mutations in primary human hematopoietic stem and progenitor cells (HSPCs). To this aim, we have designed and tested four different guide RNAs (gRNAs) targeting the start codon of the WAS gene in haematopoietic cell lines. Upon delivery of each gRNA complexed with Cas9 protein, we were able to achieve high frequency of gene editing. The best performing Cas9/gRNA complex was further tested in human HSPCs from different donors, reaching up to 80% of editing efficiency. To deliver the donor DNA molecule which serves as a template for

HDR-mediated repair, we created an Adeno Associated virus (AAV) that contains a PGK promoter-driven GFP cDNA flanked on each side by ~800bp-long WAS homology arms. Delivery of both Cas9/gRNA complex and AAV donor template resulted in the knock-in of the GFP cassette in up to 46% of HSPCs, with high rates of primitive hematopoietic stem cells editing. Edited HSPCs retained their potential to give rise to multiple lineages in colony forming unit assays, without lineage skewing. We then created a therapeutic targeting vector by replacing the PGK-GFP cassette in the AAV6 donor construct with a promoterless codon optimized WAS cDNA. Delivery of the Cas9/gRNA complex and the therapeutic AAV6 donor to WAS-deficient HSPCs and T cells derived from WAS patients led to the restoration of WAS expression at levels comparable to those of wild-type cells, and to the correction of their functional defects. We are currently assessing the long-term engraftment and differentiation potential of corrected HSPCs in animal models, to determine the best editing strategy and establish a viable therapeutic approach to treat WAS deficiency.

Musculo-Skeletal Diseases I

7. Aspiro Phase 1/2 Gene Therapy Trial in X-Linked Myotubular Myopathy (Xlmtm): Preliminary Safety and Efficacy Findings

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X-linked myotubular myopathy (XLMTM) is a rare monogenic disease caused by mutations in the *MTM1* gene and characterized by profound muscle weakness, respiratory failure and early death. A retrospective chart review of 112 boys with XLMTM (RECENSUS study [Beggs, 2017]) showed overall mortality of 44% (mortality occurred in 64% of patients aged ≤18 months and in 32% of patients aged >18 months). Additional natural history data are being collected in an ongoing, prospective, non-interventional, run-in study of male patients with XLMTM (the INCEPTUS study), to serve as a longitudinal baseline and within-patient control for the ASPIRO clinical study. Here we report early data from Cohort 1 of ASPIRO, a Phase 1/2, open-label, randomized, ascending dose study to evaluate the safety and preliminary efficacy of an investigational gene therapy product (AT132) in patients with XLMTM. AT132 (rAAV8-Des-hMTM1) is designed to deliver functional copies of the *MTM1* gene to skeletal muscle cells. Approximately 12 XLMTM patients less than 5 years of age are planned to be randomized into three ascending dose cohorts (n=4 per cohort) to receive a single AT132 administration (n=3) or to act as a delayed treatment control (n=1). Treatment-randomized Cohort 1 patients received an intravenous infusion of $1x10^{14}$ vector genomes (vg) per kg of AT132. At time of data cut (21Dec17), individual patient follow-up ranged from 4 to 12 weeks. A total of 6 adverse events (AEs) were reported, of which 3 were deemed probably or possibly related to drug and 2 were deemed serious AEs (SAEs). Both SAEs occurred in Patient 3, who is responding to intravenous steroids and supportive care. Efficacy assessments have shown notable improvements in both the Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP-INTEND) scale and maximal inspiratory pressure (MIP) (Table 1). Whereas age-appropriate motor milestones had not been achieved at baseline, by week 12, Patient 1 (aged 1 year) had acquired several new developmental skills, including the ability to control head movements, roll over by himself and sit unassisted for >5 seconds. Patient 2 was observed for 8 weeks, and Patients 3 and 4 (Control) were observed for 4 weeks. All treated patients have demonstrated improvements in airway clearance and secretion management as evidenced by changes in the Parental Global Impression of Secretion Improvement and Severity Scales. Clinical Global Impression of Improvement reports demonstrate increased limb and trunk strength, improved velocity and accuracy of movement and increased loudness during vocalization and crying, improving their ability to communicate. Updated safety and efficacy data will be presented at the ASGCT 21st Annual Meeting.

Table 1: ASPIRO Cohort 1 Baseline Characteristics and Preliminary Efficacy Results

Patient #		1	2	3	4 (Delayed Treatment Control)
Age at Baseline (Years)		0.8	4.1	2.6	4.0
Ventilator Support		12 h/day BiPAP	17 h/day invasive ventilation	24 h/day invasive ventilation	12 h/day BiPAP
CHOP-INTEND Score (a maximum score of 64 is typically attained in healthy infants 3-6 months of age)	Median Score in INCEPTUS	29	45	28	49
	Baseline Score in ASPIRO	29	45	34	49
	Most Recent Score in ASPIRO	56 (week 12)	56 (week 8)	36 (week 4)	46 (week 4)
	Change from Baseline (%)	27 (93%)	11 (24%)	2 (6%)	-3 (-6%)
Maximum Inspiratory Pressure (cm H_2O; normal minimal pressures in healthy children <5 years are ≥ 80 cm H_2O)	Median Pressure in INCEPTUS	29	34	24	65
	Baseline Pressure in ASPIRO	33	44	26	58
	Most Recent Pressure in ASPIRO	80 (week 12)	77 (week 4)	44 (week 4)	NA
	Change from Baseline (%)	47 (142%)	33 (76%)	18 (70%)	NA

8. AAV Micro-Dystrophin Therapy Ameliorates Muscular Dystrophy in Young Adult Duchenne Muscular Dystrophy Dogs for Up to Thirty Months Following Injection

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Duchenne muscular dystrophy (DMD) is a lethal inherited childhood muscle disease caused by dystrophin deficiency. An effective gene therapy for DMD requires efficient whole body muscle transduction. Canine DMD (cDMD) is by far the best large animal model for DMD. We recently demonstrated that systemic administration of vectors derived from adeno-associated virus-9 (AAV-9) leads to safe and bodywide gene transfer in cDMD for four months. Here we evaluated the long-term safety and therapeutic efficacy of AAV-9 micro-dystrophin gene therapy in cDMD. Five 2.5 to 3.5-m-old affected dogs were treated at doses of 0.5 (n=1, low), 1 (n=2, medium), 3 (n=1, high) and 5 (n=1, high) $x10^{14}$ viral genome particles/kg. All dogs received transient immune suppression and tolerated the infusion with no adverse events. Comprehensive blood profile analysis was unremarkable for up to three years. Muscle biopsy revealed sustained micro-dystrophin expression up to the last biopsy time point (30 months for the low and medium doses and 20 months for the high dose). Muscle micro-dystrophin expression was confirmed bodywide in a medium dose dog necropsied at 8 months post-injection. AAV micro-dystrophin therapy restored the dystrophin-associated glycoprotein complex and mitigated inflammation, fibrosis and calcification. No deterioration of activity was observed in treated dogs. The average growth rate showed a trend of normalization in treated dogs and limb muscle force was significantly enhanced. A single muscle force assay in the necropsied dog showed significant improvement compared to that of untreated dogs. Our results suggest that systemic AAV micro-dystrophin therapy is safe and effective in treating large mammal models for DMD. Furthermore, it supports the development of microgene vectors for DMD clinical trials.

9. Dose Escalation Study of Systemically Delivered AAVrh74.MHCK7.Micro-Dystrophin in the Mdx Mouse Model of DMD

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Introduction: Duchenne muscular dystrophy (DMD) is the most common severe childhood form of muscular dystrophy. More than 2000 mutations of the *DMD* gene are responsible for progressive loss of muscle strength, loss of ambulation, and ultimately respiratory and cardiac failure. There is an unmet need for DMD therapies that target the root cause of disease while applying to a variety of mutations. The number of observed and theoretical mutations poses challenging problems considering evolving therapies such as CRISPR/Cas9 and gene replacement therapy. Immunity to both adeno-associated virus (AAV) and transgene must be considered and strategies for cardiac gene restoration must be considered. The study to be presented demonstrates efficacy following systemic delivery of rAAVrh74.MHCK7.micro-dystrophin in a dose dependent manner. Viral titers were equal to or higher than those shown to be safe and effective in spinal muscular atrophy (N Engl J Med 2017;377:1713-22). Pre-clinical studies include both mdx mice and non-human primates, providing a path for clinical development of microdystrophin gene therapy in DMD. **Methods:** We designed an AAVrh74 vector containing a codon optimized human micro-dystrophin transgene driven by a muscle-cardiac specific promoter, MHCK7. To test efficacy of rAAVrh74.MHCK7.micro-dystrophin, we evaluated systemic injections via the tail vein in mdx (dystrophin null) mice in a dose ascending schedule designed to reverse the clinical disease. The three doses included were low ($8x10^{13}$ vg/kg), mid ($2x10^{14}$ vg/kg), and high dose ($6x10^{14}$ vg/kg). **Results:** The results of this study demonstrate that systemic delivery of AAVrh74.MHCK7.micro-dystrophin is efficacious in normalizing histologic and functional outcome measures in a dose dependent manner in the mdx mouse. Specific force output increased in the diaphragm and the tibialis anterior muscle, with mid and high doses eliciting force outputs no different than wild-type levels. Additionally, there was a more normalized muscle environment demonstrated by reductions in centralized nucleation and normalized myofiber diameters with systemic delivery. Finally, in the non-human primate, systemic delivery at 2×10^{14} vg/kg elicited widespread gene expression through immunofluorescence staining and western blot. There was no significant vector-associated toxicity reported either by clinical or organ specific laboratory assessments or following formal histopathology reviewed by a board-certified veterinary pathologist. Both assessments revealed no abnormalities in the mdx mice or non-human primates. **Conclusions:** The findings in this non-clinical study provided proof of principle for safety and efficacy of systemic delivery of AAV.micro-dystrophin at high vector titers supporting initiation of a Phase I/II safety study in DMD boys.

10. Gene Therapy Leads to Dose-Dependent Transcriptome Remodeling and Provides Biomarkers of Therapeutic Efficacy in X-Linked Myotubular Myopathy

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Multiple clinical trials using recombinant adeno-associated virus (rAAV) vectors have been initiated for neuromuscular disorders (NMDs), including Duchenne, Becker, and Limb-Girdle muscular dystrophies, spinal muscular atrophy, and recently X-linked myotubular myopathy (XLMTM). Previous work from our laboratory on a canine model of XLMTM has shown that a single rAAV injection

restores muscle function and corrects structural transverse tubules defects, with dogs surviving more than 5 years post gene transfer. This unparalleled therapeutic efficacy offers the opportunity to assess the complexity of NMD correction at the molecular level. XLMTM results from mutations in the *MTM1* gene and causes severe hypotonia associated with histological signs of muscle hypotrophy, including myofibers with small diameters and centrally positioned nuclei. *Mtm1* mutant mice show dysregulation of specific genes involved in muscle growth, atrophy, excitation-contraction coupling, and stress responses to misfolded proteins, but a comprehensive characterization of the XLMTM transcriptome is missing. In addition, the impact of gene therapy on the transcriptome is rarely investigated, even though it could help us understand the underlying mechanisms of disease rescue, identify molecular pathways escaping correction, and provide new biomarkers to predict treatment efficacy and monitor durability. The present study interrogates the mechanisms of XLMTM correction at the transcriptome level, in a dog cohort showing dose-dependent clinical improvements after rAAV8-cMTM1 gene transfer. We showed that RNA sequencing (RNA-seq) successfully discriminated dogs treated with a therapeutic dose of rAAV from those injected with a sub-therapeutic dose. Our analysis confirmed several dysregulated genes previously observed in XLMTM mice, but also identified new genes linked to specific disease phenotypes. Importantly, rAAV8-cMTM1 gene transfer leads to XLMTM transcriptome remodeling and dose-dependent normalization of gene expression levels. To compare the impact of each rAAV dose, we created an analytical framework composed of visualization tools and performance indicators based on gene expression patterns and measurable from RNA-seq data (Figure 1). Finally, we propose a list of potential clinically relevant RNA biomarkers to be validated in future studies testing the efficacy of gene therapy protocols for XLMTM. In conclusion, our study describes an innovative approach for the analysis of rAAV-treated muscles using transcriptomic data, and sheds light on the molecular mechanisms associated with both the pathological development of XLMTM and its correction post gene therapy.

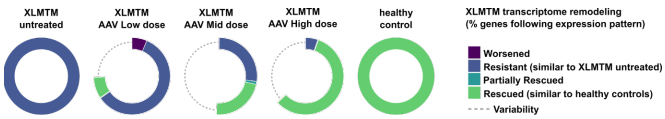

Figure 1: transcriptome remodeling in the *Biceps femoris* of rAAV-treated XLMTM dogs. The expression patterns are detailed on the right-hand side and color-coded. The arc lengths are proportional to the percentages of genes whose expression follows the associated patterns when compared with untreated XLMTM dogs and healthy controls.

11. AAV-SERCA2a Gene Therapy Ameliorated Muscle Disease in Duchenne Muscular Dystrophy Dogs

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Duchenne muscular dystrophy (DMD) is a severe and progressive muscle disorder caused by mutations in the *dystrophin* gene resulting in the loss of the cytoskeletal protein dystrophin. Compromised sarcolemmal integrity followed by Ca^{2+} influx and cellular necrosis characterize muscle pathology in DMD. Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) is a sarcoplasmic reticulum (SR) calcium pump that plays a crucial role in regulating intracellular calcium concentration by actively transporting Ca^{2+} from the cytosol into the SR. It has been shown that SERCA expression and activity are reduced in dystrophic muscle. Here we tested the hypothesis that adeno-associated virus (AAV) mediated SERCA2a gene transfer can ameliorate muscle disease in the canine DMD model. 7.83×10^{13} viral genome particles of Y731F tyrosine mutant AAV-9 SERCA2a vector were injected into the extensor carpi ulnaris muscle of young adult, affected dogs under transient immunosuppression. Treated muscles showed widespread transgene expression upon examination 12 weeks later. AAV SERCA2a treatment normalized SERCA2a protein expression and improved the impaired SR Ca^{2+} reuptake. Furthermore, AAV treatment restored the expression levels of SR calcium handling proteins. SERCA2a overexpression significantly mitigated histological markers of muscle disease such as central nucleation, fibrosis and degeneration. Treatment also normalized myofiber type distribution. Importantly, AAV SERCA2a therapy significantly protected the muscle from eccentric contraction induced force loss. Our results suggest that targeting intracellular Ca^{2+} regulation via AAV SERCA2a therapy has significant therapeutic potential for DMD.

12. A Phase 1/2 Clinical Trial of Intra-Arterial Gene Transfer of rAAVrh74.MCK.GALGT2 for DMD: Initial Safety Profile

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Duchenne Muscular Dystrophy (DMD) is an X-linked progressive disease characterized by the loss of dystrophy protein as a result of truncating mutations in the *DMD* gene. Expression of the β1,4 N-acetylgalactosaminyl transferase encoded by *GALGT2* (or *B4GALNT2*) is typically localized only to two muscle membrane

domains, the neuromuscular junction and the myotendinous junction. In the mdx mouse model of DMD, overexpression of *GALGT2* results in improvement of muscle pathology and function comparable to dystrophin restoration therapies, and regional infusion via intra-arterial delivery leads to significant transduction of leg muscles in non-human primates. These results that have led to a first-in-human trial (NCT03333590). The first patient enrolled, an 8 year old boy with DMD, received $2.5x10^{13}$ vg/kg per leg ($5x10^{13}$ vg/kg total) by isolated limb infusion, in which femoral venous and femoral arterial blood flow is stopped and the vector instilled into the femoral artery for a 10 minute dwell time. The procedure was well-tolerated; serum transaminases (elevated due to DMD) remained within pre-treatment range, and no regional or systemic signs of toxicity were seen through Day 60 post-gene transfer. Functional assessments and repeat muscle biopsy in the first subject await his Day 90 visit, and enrollment in the study is ongoing.

Cancer - Oncolytic Viruses

13. The Innate Immune System as a Determinant for Response to Measles Virotherapy

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Despite documented successes using oncolytic viruses (OV) to treat cancer; clinical outcomes vary significantly, reflecting variability in virus replication. We hypothesized that virus replication in tumor cells is determined by restriction mechanisms following viral entry. Using Edmonston vaccine derived oncolytic Measles virus (MV) strains, we tested this by performing RNA-Seq analysis to identify differentially activated pathways between MV permissive (GBM43 and GBM64) and resistant (GBM39, GBM150 and GBM6) primary tumor lines, in the context of comparable viral entry receptor levels. The type I interferon (IFN) pathway was constitutively and differentially active in MV resistant lines (p-value= $2.1x10^{-20}$), and characterized by elevated baseline expression levels of interferon-stimulated genes (ISGs). We developed a Diagonal Linear Discriminant Analysis (DLDA) prediction algorithm using a unique set of ISGs and successfully applied it to 35 patient derived orthotopic GBM xenografts and 86 ovarian cancer (OvCa) avatars. A randomly selected GBM line predicted to be permissive to MV therapy (GBM10) was implanted into mice and challenged with inactivated MV or MV. Mice implanted with GBM10 cells demonstrated a significant prolongation in survival (24 vs 39 days, respectively, p value< 0.001) whereas mice implanted with a predicted MV resistant line (GBM76) demonstrated no difference in survival (89 vs 85 days, p-value= 0.1324). We further validated our prediction algorithm across tumor types using primary patient derived OvCa avatars. Mice implanted with a line predicted to be MV permissive demonstrated a significant prolongation in median survival upon MV therapy (22 days, p-value= 0.01), whereas mice implanted with a predicted MV resistant line had no therapeutic benefit (85 vs 89 days, p-value= 0.48). Our DLDA algorithm was then successfully validated in 10 consecutive glioblastoma patients treated with an oncolytic MV strain, as part of an ongoing clinical trial. Patients had tumor resection on day 5 following viral administration, allowing us to evaluate virus replication by qRT-PCR and correlate it to the DLDA score. Virus replication within the treated tumors was inversely correlated with elevated levels of ISG expression and DLDA score (ρ= -0.717; p-value= 0.03). Our scoring system identified a class of patients with intermediate DLDA scores and moderate levels of virus replication. These patients could likely benefit from MV therapy in combination with drugs that inhibit the IFN pathway. To test this, we utilized the FDA approved JAK/STAT inhibitor, Ruxolitinib. ISG expression is significantly reduced in the MV resistant GBM39 line using Ruxolitinib (3μM) for 48 h *in vitro*. Treatment with Ruxolitinib prior to MV therapy treatment sensitizes resistant GBM cells to killing and significantly increases virus production (1000-fold, p-value= 0.03). Our results represent the first report of a predictive algorithm that can identify patients likely to respond to oncolytic virus therapy. The genes in our panel encode proteins with known antiviral functions against other oncolytic viruses currently being tested clinically, thus this predictive signature could have widespread use. Funding: Brain SPORE (P50 CA108961), R01 CA154348

14. Combined Mesothelin-Redirected Chimeric Antigen Receptor T Cells with Cytokine-Armed Oncolytic Adenoviruses for the Treatment of Pancreatic Cancer

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Pancreatic ductal adenocarcinoma (PDA) is characterized by its highly immunosuppressive tumor microenvironment (TME) that can limit T cell infiltration and induce T cell hypofunction. Mesothelin-redirected CAR T cell (meso-CAR T cell) therapy has shown feasibility and some efficacy in clinical trials but antitumor efficacy remains limited. We tested the hypothesis that combined meso-CAR T cell therapy

with an oncolytic adenovirus expressing TNF-α and IL-2 (Ad5/3-E2F-D24-TNFa-IRES-IL2 or TILT-123) would improve efficacy in syngeneic and xenogeneic mouse pancreatic tumor models. Ad5/3-E2F-D24-TNFa-IRES-IL2 enhanced the anti-tumor efficacy of human meso-CAR T cells in immune-deficient mice engrafted with human PDA, and was associated with robustly increased tumor infiltrating lymphocytes (TILs) and enhanced CAR T cell function. Importantly, the combined therapy prevented metastasis in contrast to either therapy alone, indicating a systemic effect of the intratumorally injected Ad5/3-E2F-D24-TNFa-IRES-IL2 in combination with intravenously injected meso-CAR T cells. Combining Ad5/3-E2F-D24-TNFa-IRES-IL2 with meso-CAR T cells also downregulated mesothelin expression in tumors, indicating enhanced on-target CAR T cell activity. Further analyses of TILs at later phases of treatment revealed that Ki67-positive TILs were associated with sustained tumor regression. We also evaluated this approach in a syngeneic mouse tumor model by combining adenovirus expressing murine TNF-α and murine IL-2 (Ad-mTNFa-mIL2) and newly established mouse CAR T cells. This novel approach induced significant tumor regression in mice engrafted with highly aggressive and immunosuppressive PDA tumors, whereas even multiple dosing of CAR T cells alone failed to suppress tumor growth. Ad-mTNFa-mIL2 increased both CAR T cell and host T cell infiltration to the tumor and altered host tumor immune status, marked by M1 polarization of macrophages and increased dendritic cell maturation. Moreover, Ad-mTNFa-mIL2 treatment elevated chemokine levels in the TME, which could attract immune cells. These findings indicate that combining cytokine armed-oncolytic adenovirus to enhance the efficacy of CAR T cells is a promising approach to overcome the immunosuppressive TME by inducing both CAR-dependent and CAR-independent host immunity for the treatment of pancreatic cancer.

15. Phase I Study of Potentially "Best-in-Class" Survivin-Responsive Conditionally Replicating Adenovirus for Advanced Sarcoma Actually Demonstrates Potent and Long-Term Efficacy and High Safety

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[**Background**] Recent approvals of oncolytic virus (OV) by FDA and EMA attract the worldwide oncologists and anticancer market. We developed the first platform technology to efficiently construct diverse types of a next-generation conditionally replicating adenovirus (CRA), *i.e.*, CRAs that target and/or treat tumor cells with multiple factors (m-CRAs), which can increase tumor specificity (*i.e.*, safety) and efficacy (*Gene Ther* 2005). Using this platform technology, we have generated and tested numbers of m-CRAs as anticancer agents, and found that one of the best was survivin-responsive m-CRAs (Surv.m-CRAs). In preclinical studies, Surv.m-CRAs induced more potent cytotoxic effects against most of malignant tumors than other competing CRAs, and exhibited not only stronger but also more cancer-selective phenotypes than telomerase reverse transcriptase (Tert)-responsive m-CRAs (*Cancer Res* 2005). Moreover, Surv.m-CRAs induced increased effectiveness against cancer stem cells, which are resistant to conventional therapies (*J Trans Med* 2014). Preclinical analyses suggested that Surv.m-CRA is the "best-in class" CRA. [**Trial protocol**] We submitted an IND in March, 2016 and have been performing the first-in-human investigator-initiated ICH-GCP clinical trial for refractory malignant bone and soft tissue tumors (Phase I). Three doses (low: 1×10^10 viral particle [vp], mid: 1×10^11 vp, high: 1×10^12 vp) are planned. Adverse effects were graded according to CTCAE v4.0. Viral shedding into blood, urine and saliva was monitored by PCR. Efficacy was assessed by CT according to RECIST and Choi criteria. [**Results**] Three patients underwent a single intratumoral injection of low dose Surv.m-CRA-1. Treatment-related adverse events included asymptomatic and temporal leukopenia (grade 3, n=1), CPK elevation (grade 1, n=1), fever (grade 1, n=1) or other mild events (no grade 4 events). Viral shedding into saliva was found in the first case only at 3 hours after injection and was negative in other two cases. Clinical efficacy for target lesion was observed in all three cases (PR or better). First case showed PR by Choi criteria at 4 weeks and PR by RECIST (reduction rate, 50%) at 28 weeks; surprisingly the antitumor efficacy continued for more than one year. In the second case, tumor necrosis (PR by Choi criteria) was found at 4 weeks and subsequently, bone formation (beneficial remodeling) was observed at 12 weeks after the injection. Since no DLT was observed in the low dose cohort, we started mid dose treatment. [**Conclusions**] First-in-human trial of Surv.m-CRA-1 demonstrated drastic and long-term antitumor effects only by a single injection of only 1/100 of the predicted maximal dose. Thus, the potential of "best-in-class" Surv.m-CRA-1 is, at least in part, being reproduced in clinical trial. After finishing the phase I study, we will proceed Phase I/II study of multiple injections for advanced solid tumors.

16. Preclinical Evaluation of NIS Expressing Oncolytic Adenoviruses as a Theranostic Tool for Pancreatic Cancer

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Oncolytic viruses encoding the sodium-iodide symporter (NIS) is an attractive approach to achieve radionuclide imaging of cancer. However, the potential of virus-induced radiotherapy has not been fully explored. We have developed a tumor-selective NIS-expressing oncolytic adenovirus (OAd) for therapy and imaging of pancreatic adenocarcinoma (PDAC). In our original vector (Ad5/3-Cox2-ΔE3-NIS-ADP) enhanced oncolysis was mediated by overexpression of adenoviral death protein (ADP). Although this structure was operative in detection and therapeutic regimens, we were concerned that ADP may affect NIS membrane localization, and diminish its effectiveness as a theranostic tool. We therefore designed an identical ADP-deleted virus {ADP(-)} and assessed the impact on virus efficacy. To evaluate the therapeutic potential of ADP(-), we treated mice bearing PDAC

tumors with virotherapy alone or in combination with ^{131}I. ADP(-) in combination with ^{131}I further reduced PDAC growth when compared to ADP(-) or ^{131}I alone and outperformed that with ADP(+) or AdCMV-NIS (used as a control vector, replication-deficient and in clinical trials for prostate cancer). ^{131}I uptake in PDAC tumor showed a clear trend where tissues taken from ADP(-) mice retained the highest ^{131}I uptake. SPECT/CT imaging of $^{99}mTc^{O4-}$ uptake was applied to visualize PDAC in mice using Patient Derived Xenografts (PDX) in two independent dose escalation studies. A single intratumoral dose of ADP(-) resulted in significantly greater accumulation of $^{99}mTc^{O4-}$ than ADP(+) as early as 2 days, was maintained up until 32 days and outlasted ADP(+) and AdCMV-NIS. Histological analyses of ADP(-)-infected PDX showed a distinct NIS cell membrane distribution pattern as it co-localized with cell membrane bound-cytokeratin4. Unlike ADP(-), ADP(+) produced a punctate NIS staining pattern, with little to no cell membrane localization. These results support our hypothesis that ADP-cytolytic effect reduces radionuclide uptake, rendering ADP(-) as the lead vector for NIS-based radiotherapy and imaging. We have previously established that pigs are currently the only hosts capable of supporting binding and replication of Ad3-retargeted OAds. To investigate the safety of NIS vectors, Yorkshire pigs were injected systemically with 5 x 10^{11} viral particles. Primary organ biodistribution (collected post 1 and 7 days), hematology parameters and blood chemistry (0, 1, 6 hours, day 1, 2, 3, 4, 7 post infection) were determined. Necropsy and histology evaluation, showed no adenovirus related toxicity when compared to vehicle-treated pigs (n=3). The white blood count increased at 48 hours but returned to baseline at 4 days in all animals. Blood chemistry changes were present at all time points, however no significant changes occurred over the course of the study. To assess liver and kidney dysfunction, we compared liver and kidney specific enzymes and damage markers in serum. Compared to control, Ad5/3-NIS administration did not significantly alter liver (AST, LDH, ALT, SDH) or kidney (BUN, creatinine, CK) function since values were similar- to control pigs and within normal range. To further analyze OAd biodistribution, a detailed analysis of viral DNA was performed in pig liver (9 regions), lung (6 lobes), spleen, and lymphatic nodes. Viral DNA, determined per 1 mg of tissue, was detected in lungs, spleen, lymph nodes and kidney on day 1 but not day 7. The level of viral DNA in liver was negligible as compared to lung. Together, these studies demonstrate the efficacy and safety of Ad5/3-NIS-based radiotherapy and imaging for pancreatic cancer.

17. Oncolytic Measles Virus Replication is Enhanced in Acute Lymphoblastic Leukaemia-Associated Mesenchymal Stromal Cells Which Have Adopted a Cancer Associated Fibroblast Phenotype and Correlates with Upregulation of Matrix Metalloproteinase 1

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There is increasing evidence that the microenvironment in acute lymphoblastic leukaemia (ALL) plays a key role in treatment resistance and relapse. There is evidence that alpha-smooth muscle actin (αSMA) positive mesenchymal stromal cells (MSCs) form a protective niche with the few surviving ALL cells post chemotherapy, leading to treatment resistance and relapse. Oncolytic viruses (OV) are emerging as potential cancer therapeutics. Clinical trials have led to the FDA approval of an OV for treatment of advanced melanoma. OVs were recently shown by Ilkow et al to utilise cross-talk between the fibroblasts in the tumour microenvironment - often termed as cancer associated fibroblasts (CAFs) - and cancer cells for enhanced viral replication. Tumour cells produced TGFβ1 that re-programmed CAFs rendering them sensitive to virus infection. Our lab and others have shown measles virus (MV) to be a promising OV for ALL therapy *in-vivo*. Given the αSMA positive MSCs in the ALL treatment resistance niche resemble CAFs, we hypothesised that MV may be a suitable agent to selectively target MSC with a CAF phenotype and therefore overcome niche mediated protection and potentially enhance ALL cell killing. We developed two models of CAF induction - using MSC cell line HS27a exposed to either an ALL cell line SD1 or TGFβ1. Both TGFβ1 and SD1 cell exposure induced HS27a cells to attain a CAF phenotype (assessed by imaging for αSMA/F-actin and gene expression profiling) which in turn were more permissive to MV replication (as assessed by MVN mRNA levels) as compared to untreated HS27a. Next, we sought to determine if primary MSCs taken from bone marrow specimens of patients enrolled in the UKALL14 trial in ALL exhibited a CAF phenotype and if so, the impact on MV replication. Among all the patient specimens tested, a proportion of MSCs had a CAF phenotype, indicated by imaging and gene expression profiling. We found 1-2 logs enhanced MV replication in both diagnostic (N=3) and post treatment (N=3) MSC as compared to healthy donor MSCs. The most highly upregulated gene in both the primary patient-derived CAF and SD1-induced HS27a-derived CAF was matrix metalloproteinase 1 (*MMP1)* (1.5 to 6300-fold higher than the control). MMP1 has previously been shown to degrade components of the extra cellular matrix (ECM), thereby helping distribution and efficacy of oncolytic virus. We showed that the degree of upregulation of *MMP1* closely related to MVN mRNA level (p=0.0355, paired t test). Thus, MSC with a CAF phenotype, which we have previously shown to be important in maintaining the acute lymphoblastic leukaemia "niche", may be attractive targets for oncolytic MV. Given that the degree of MV-replication appears to correlate closely with MMP1 expression, chemotherapy agents such as cytarabine, that we have already shown to upregulate MMP1 expression, may be an effective combination with MV to target the ALL niche which is important in therapy resistance.

18. Oncolytic Viruses to Enhance BiTE and CAR Therapy of Solid Tumors

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Bispecific T cell engagers (BiTEs) and chimeric antigen receptors (CARs) redirect T cells to tumor surface markers and have shown promising results in the treatment of hematological malignancies, but so far lack clinical efficacy in solid tumors at acceptable levels of toxicity. We hypothesize that oncolytic virotherapy will increase efficacy of these approaches by supporting tumor cell lysis, reversing tumor immunosuppression and enhancing accessibility of tumor cells and tumor antigens (Fig. 1). Oncolytic measles vaccine strain viruses (MV) encoding BiTEs (MV-BiTE) mediate secretion of functional BiTEs from infected tumor cells. In a syngeneic melanoma model, intratumoral MV-BiTE injections significantly prolonged survival compared to unmodified virus, virus encoding a control BiTE, and purified BiTE alone. After MV-BiTE treatment, we observed increased intratumoral effector T cells and confirmed protective anti-tumor immune memory in long-term survivors. Immune transcriptome analysis was performed to identify underlying mechanisms. In patient-derived xenograft models, combined treatment with MV-BiTE and human PBMCs significantly prolonged survival compared to either monotherapy alone. Pharmacodynamic analyses revealed intratumoral BiTE expression while serum levels remained below detection limit, indicating successful local delivery while preventing systemic exposure. We are currently testing combined MV and CAR T cell therapy approaches to further enhance efficacy and specificity and to broaden the repertoire of available treatments. First *in vitro* and *in vivo* applications indicate therapeutic benefit and potential synergy. We have generated a mathematical model of these combinations accounting for both oncolytic and immunological effects that will be applied to identify optimal parameters for effective treatment. Conclusively, our data support rational choices for successful combinatorial treatment regimens and highlight the potential of OV-mediated improvement of immunotherapeutic strategies.

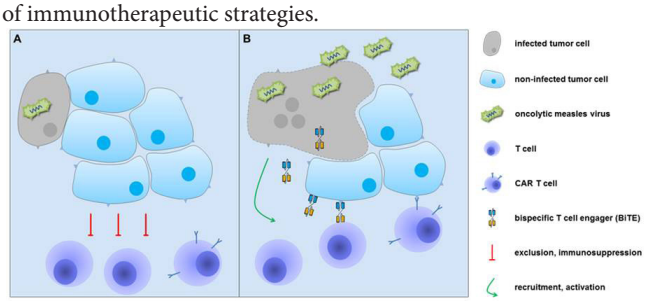

Fig. 1: Oncolytic support for BiTEs and CARs. Infection of susceptible tumor cells (A) induces viral replication, tumor cell lysis and local inflammation, generating a favorable environment for T cell-based therapies (B).

Cancer - Targeted Gene & Cell Therapy I

19. Monocyte-Derived IL-1 and IL-6 are Differentially Required for Cytokine Release Syndrome and Neurotoxicity by CAR-T Cells

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Background. The remarkable antileukemia efficacy by CD19-specific CAR T cells reported so far in humans is frequently associated with life-threatening cytokine release syndrome (CRS) and neurotoxicity. Unfortunately, all these issues cannot be addressed pre-clinically in currently available NSG mouse models, because they lack human hematopoiesis and, furthermore, ultimately develop xenograft-versus-host disease (X-GVHD), preventing the evaluation of long-term effects. **Methods**. T cells reconstituting in NSG mice transgenic for human stem cell factor (SCF), IL-3 and GM-CSF (SGM3) after transplantation with human HSCs were CAR-engineered *ex vivo* with either a CD19 or a CD44v6 CAR (both having a CD28 2G design) after activation with CD3/CD28-beads and IL-7/IL-15 and infused into secondary recipients co-engrafted with autologous human HSCs and leukemia. **Results**. SGM3 mice reconstituted high levels of human T cells, which, once transferred in secondary recipients, persisted up to 200 days without causing X-GVHD, even after irradiation. Robust and specific xeno-tolerance was confirmed by *in vitro* hyporesponsiveness to NSG, but not to C57/Bl6 antigens or human HLAs (PBMCs). When HuSGM3 CAR-T cells were infused in humanized (Hu) leukemic SGM3 secondary recipients, tumor clearance resulted in the development of a clinical syndrome similar to CRS observed in clinical trials, and characterized by high fever, elevated systemic human IL-6 and serum amyloid A levels - mouse analog of C-reactive protein in humans. As demonstrated *in vivo* by single-cell RNA sequencing and flow cytometry, human monocytes were major sources of IL-1 and IL-6 during CRS, where IL-1 always preceded IL-6 production ($P<0.001$). CRS lethality was similar between mice infused with CD19 CAR-T cells or CAR-T cells specific for CD44v6, a target antigen expressed on both leukemia and monocytes. Strikingly, mice recovering from CRS benefited from durable leukemic remissions, yet experienced long-lasting CD19+ B-cell or CD44v6+ monocyte aplasias. Interestingly, in this model, either prophylactic or therapeutic tocilizumab administration abolished CRS by CD19 or CD44v6 CAR-T cells but without interfering with their comparable and long-term anti-leukemic effects. Conversely, depleting monocytes/macrophages by either liposomal clodronate or by the prophylactic CD44v6 CAR-T cells inhibited CRS development, but also resulted in significantly worse leukemia-free survival (at 250d, 0% vs 80%,

P<0.0001). Despite preservation of antileukemia efficacy, tocilizumab administration failed to protect mice from delayed lethal neurotoxicity, characterized by meningeal inflammation at histopathology. Instead, administering IL-1 receptor antagonist anakinra abolished both CRS and neurotoxicity, resulting in significant prolongation of survival in the absence of leukemia. **Conclusion**. Our results indicate that monocytes are required for both optimal anti-leukemic efficacy and CRS development, although we hypothesize they exert their dual function through different mechanisms. The information obtained in this study might be key for the design of safer engineered cell therapies in cancer and other diseases.

20. Turbocharged CAR T Cells Stimulate Host T Cells against Prostate Cancer

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Both CD28- and 4-1BB-based second-generation CAR T cells elicit dramatic clinical responses in patients with refractory/relapsed CD19 positive malignancies, especially patients with acute lymphoblastic leukemia. However, due in part to their tumor microenvironment and tumor heterogeneity, solid tumors often resist CAR T cell therapy. We demonstrated that co-expressing the second-generation 19-28z CAR with 4-1BBL yields balanced tumoricidal function and T cell persistence, resulting in greater therapeutic efficacy (Turbocharged CAR). We hypothesized that CD28-based second-generation CAR T cells coexpressing 4-1BBL would have better therapeutic efficacy against solid tumors than current second-generation CARs, owning to their unique intrinsic and immunomodulatory qualities. Prostate-specific membrane antigen (PSMA) is a dimeric type II integral membrane glycoprotein, which is overexpressed in castrate-resistant, metastatic prostate cancer. We constructed a tricistronic PSMA-targeted CAR vector encoding the Pd28z CAR, 4-1BBL and a truncated, nonfunctional EGFR as a safety control (Pd28z-4-1BBL-EGFRt). Two second-generation CARs (Pd28z and PdBBz) served as controls. To demonstrate that CAR T cells may stimulate tumor specific T cells, we generated engineered NY-ESO-1 specific T cells with CRISPR/CAS9 technology. In a homogeneous $PSMA^+$ prostate cancer model (100% $PSMA^+$) of late stage disseminated cancer, we used the in vivo "stress test" in which the T cell dose is gradually lowered to levels where CAR therapy begins to fail, in order to compare the relative functionality and persistence of CAR T cells. CAR T cells coexpressing Pd28z with 4-1BBL exhibited complete tumor eradication and stronger T cell persistence in NSG mice bearing diffuse metastatic prostate cancer, compared to both second-generation CARs Pd28z and PdBBz. In a heterogeneous prostate cancer model (90% $PSMA^+$), 4-1BBL Turbocharged CAR T cells demonstrated better efficacy as than both second-generation CARs, but tumor relapsed about 50 days later. In order to test the host immune response stimulation potential of Turbocharged CAR T cells, we generated another heterogeneous prostate cancer model ($PSMA^+$ $A2^-$ NY-ESO-1^- 70%, $PSMA^-$ $A2^+$ NY-ESO-1^+ 30%) and NY-ESO-1 specific T cells. NY-ESO-1 TCR T cells show stronger tumor eradication when combined with Turbocharged CAR T cells than two second-generation CARs. Thus, CD28/CD3ζ CAR T cells that co-express 4-1BBL are poised to recruit the host immune response against the tumor, potentially diversifying the antigen specificity of the immune response beyond the CAR target antigen and stimulating immunity that outlives the CAR T cells themselves.

21. HER2-Targeted Dual Switch CAR-T Cells Enable Post-Infusion Control of CAR-T Efficacy and Safety with Small Molecules

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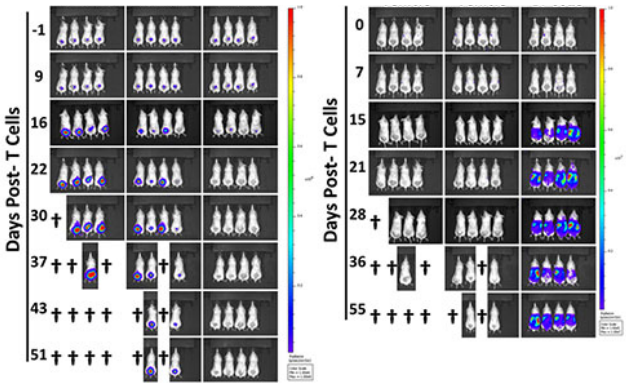
Background: Due to overexpression in many distinct solid tumors, HER2 is an attractive Chimeric Antigen Receptor-T cell (CAR-T) target; however, safety concerns from off-tumor HER2 expression necessitate greater control over such "living" drugs post-infusion. Here, we demonstrate that Dual Switch (DS) HER2-specific CAR-T cells (iRMC+iC9+HER2.ζ) engineered to express both inducible MyD88/CD40 (iRMC) costimulatory and Caspase-9 (iC9) proapoptotic elements enable ligand-specific post-infusion control of CAR-T cell expansion and resolution of toxicity. **Methods/Results:** Activated T cells were cotransduced with a first γ-retrovirus (RV) encoding the iRMC costimulatory protein and a trastuzumab-based first-generation CAR (pSFG-iRMC-HER2.ζ) in addition to a second RV encoding the iC9 safety switch and a truncated CD19 marker for MACS enrichment (pSFG-iC9-ΔCD19). In some cases, a unified vector encoding all three elements (pSFG-iRMC-iC9-HER2.ζ) was employed. *In vitro* coculture assays demonstrated that activation of iRMC with a non-immunosuppressive rapamycin analog, BPC015, efficiently eliminated $HER2^+$ cell lines OE19 (E:T,1:15) and SK-OV3 (1:10). In these cocultures, iRMC induction in DS CAR-T cells with BPC015 produced high levels of IL-2 (5571 ± 3532 pg/ml), IFN-γ (13809 ± 5348 pg/ml), and TNF-α (1020 ± 415.4 pg/ml). Rimiducid (Rim) activation of the iC9 switch rapidly induced caspase-3/7 activity (½Vmax ~3 hours) and DS CAR-T cell apoptosis. *In vivo,* $HER2^+$ OE19.GFP*luc* tumor-bearing immunodeficient NSG mice were infused with human DS CAR-T cells, and iRMC-activation with BPC015 resulted in tumor elimination. The rate of DS CAR-T expansion *in vivo* was BPC015 dose-dependent. At 1 mg/kg BPC015, activation of iRMC induced >100-fold expansion (by bioluminescent imaging) of DS CAR-T cells and concomitant increases in serum cytokines. However, acute toxicity (>10% body weight reduction) was observed at high BPC015 doses. Activation of iC9 by systemic administration of 0.5 mg/kg Rim rapidly eliminated DS CAR-T cells, reduced serum cytokine levels and resolved toxicity while retaining long-term tumor control (>60 days). Elimination of DS CAR-T cells was titratable with Rim. **Summary:** Control of Dual Switch CAR-T cell therapies post-infusion may provide a potent, yet safe clinical approach for HER2-targeting in solid malignancies. *In vivo* DS CAR-T cells could be modulated with high affinity, cell-permeable small molecules to promote CAR-T cell expansion and increase anti-tumor efficacy or to effect rapid resolution of acute-cytokine-related toxicities while retaining long-term efficacy.



22. Intracerebral Immunomodulation Using Genetically Engineered Mesenchymal Stem Cells Induces Long-Term Survival and Immunity in Glioblastoma

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Glioblastoma (GBM) represent one of the most aggressive brain tumors. Standard therapy consists of surgical removal followed by radiation and chemotherapy. Due to its invasive growth all tumors relapse, which correlates with a dismal prognosis and an average survival period of 12 to 14 month after diagnosis. Mesenchymal stem cells (MSCs) show an inherent brain tumor cell tropism that can be exploited for targeted delivery of therapeutic genes to invasive glioma. We assessed whether a motile MSC-based local immunomodulation is able to overcome the immunosuppressive GBM microenvironment and to induce an antitumor immune response. We provide preclinical data of a glioblastoma targeted cell-based immunotherapy consisting of allogeneic MSCs which are genetically modified to co-express high levels of IL12 and IL7. In vitro characterization demonstrated that MSC-IL12/7 increased T-cell activation, as measured by increased secretion of interferon gamma and tumor necrosis factor alpha. Furthermore, the MSCs promoted NK cell mediated killing of GBM cell lines in co-culture assays. MRI-based cell tracking and histological time-course analysis confirmed a rapid and targeted tumor tropism towards intracerebral glioblastoma xenografts in mice after injection to the contralateral cerebral hemisphere. Therefore, therapeutic efficacy of MSC-IL12/7 was assessed in an immunocompetent orthotopic GL261/C57Bl6 glioblastoma model. Intratumoral administration of MSC-IL12/7 at day 5 or day 10 induced a significant tumor growth inhibition and displayed a significant increase of intratumoral T2-hypointensities on MRI, suspicious for tumor necrosis. All control animals died by day 28, while 50% of treated mice survived long-term (>100d). MRI in long-term survivors after a single treatment with MSC-IL12/7 did not reveal any tumor mass and demonstrated a clear tumor regression when compared with initial MRIs. Re-challenging survivors with another intracerebral injection of GL261 confirmed long-lasting tumor immunity. Whereas naive control animals died quickly as expected, re-challenged survivors did not develop any signs of tumor growth on MRI and displayed long-term survival. Immunmodulatory effects were assessed in a separate experiment by immunhistology and flow-cytometry at different time points to comprehensively profile immune activation of tumor-infiltrating lymphocytes and peripheral blood lymphocytes. Local treatment with MSC-IL12/7 was well tolerated and led to a significant switch of CD4+/CD8+ T-cell ratios compared to control animals with an intricate predominantly CD8+ T-cell mediated anti-tumor response. Local MSC-based delivery of immunmodulatory cytokines is well tolerated and able to efficiently alter the immunosupressive microenvironment in glioblastoma resulting in tumor immunity. The significant and long lasting therapeutic effects warrant a rapid clinical translation of this concept and have led to planning of a phase I/II study.

23. T Cell Redirection with Coupled Long Terminal-CRISPR Gene Editing Effects

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Allogeneic chimeric antigen receptor (CAR) and T cell receptor (TCR) redirected T cells are constrained in their use due to HLA barriers and allo-recognition, but gene editing has uncovered opportunities to overcome these hurdles. Initial proof-of-concept applications have described the generation of 'universal' CAR T cells expressing CARs and modified with DNA-targeting nucleases to disrupt the chromosome 14q T cell receptor alpha constant chain (TRAC) locus and trials are underway to test these approaches. While efficient, transgene expression and editing effects were unlinked, resulting in variable yields of heterogenous T cell populations and complicating cell dosing strategies. We report a self-inactivating lentiviral 'terminal' vector platform that couples transgene expression with CRISPR/Cas9 scission effects through the precise incorporation of sgRNA element(s) into the ΔU3 3' lentiviral long terminal repeat (LTR). Following reverse transcription and duplication of hybrid ΔU3-sgRNA to the 5'LTR, delivery of Cas9 mRNA by electroporation resulted in highly efficient TRAC locus modification. Scaled automated magnetic bead mediated separation delivered enrichment of highly homogenous (>96%) CAR+ (>99%) TCR- populations. Molecular interrogation by multiple sequencing modalities verified on-target specificity while importantly showed no evidence of in silico predicted off-target events. Nor was evidence of 14q translocations detected. Robust anti-leukemic effects were demonstrated by serial bioluminescence in a humanized immunodeficient tumour murine model and these were better sustained in comparison groups where animals were treated with conventional CAR+TCR+ T cells. Similarly, T cell modified with recombinant T cell receptor (rTCR), could be depleted of endogenous receptors, reducing the risk of cross-pairing, autoimmunity and off target effects. Furthermore, multiplexing allowed for simultaneous

disruption of additional targets, such that dual disruption of TCR and HLA class 1 was achieved through positioning of TRAC and β2-microglobulin (B2M) specific sgRNAs under the control of huU6 and huH1 promoters within the 3' LTR. Double-knockout of 41% -65% was achieved and following magnetic bead depletion, homogenous populations of >97% CAR+ >99% TCR-MHC- were recovered. These processes are scalable and amenable to adaption for compliant manufacturing, and provide a route to clinical phase evaluation.

24. CD30-Redirected Chimeric Antigen Receptor T Cells Target CD30$^+$ and CD30$^-$ Embryonal Carcinoma via Antigen-Dependent and Fas/FasL Interactions

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Testicular germ cell tumors (TGCTs) are the most common malignancies in male adolescents and young adults, and incidence continues to rise. Embryonal carcinomas (ECs) and mixed TGCTs containing EC are the most aggressive TGCT subtypes. However, patients who relapse after chemotherapy have limited treatment options and have an overall poor prognosis. Immunotherapy may be an alternative strategy to improve overall survival while reducing chemotherapy-associated morbidities. ECs consistently express CD30, a TNF superfamily member, both at diagnosis and after relapse. To specifically target CD30, we expressed in T cells isolated from 5 healthy donors a previously clinically validated CD30-redirected chimeric antigen receptor with the CD28 endodomain (CD30.28-Ts). CAR transduction was 96±0.4% (mean±SEM). Antitumor activity was assessed against the human EC cell lines Tera-1, Tera-2, and NCCIT, which expressed CD30 (Tera-1: 68%; Tera-2: 93%; NCCIT: 76% by flow cytometry) but also contained a fraction of CD30$^{-/dim}$ cells. Compared to control (non-transduced) T cells, CD30.28-Ts exhibited potent antitumor activity *in vitro* by culture assays for Tera-1 (5.5±1.2% residual tumor, $p<0.001$) and Tera-2 (3.2±0.4, $p<0.01$) at 1:5 effector:target (E:T) ratios, while NCCIT cells were more resistant (12.5±5.6%, $p<0.01$ at 5:1 E:T ratio). Notably, CD30.CAR-Ts targeted the putative stem cell component of Tera-1 cells identified by Hoechst dye staining (n=2). CD30.28-T cytolytic activity was complemented by sustained proliferation and pro-inflammatory cytokine production. CD30.28-Ts also demonstrated antitumor activity in an *in vivo* xenograft NSG mouse model of metastatic EC, where 0.25e5 luciferase-labeled cells were engrafted orthotopically in the kidney capsule and after 15 days received i.v. either irrelevant (CD19) CAR-T or CD30.28-Ts. By day 42 post T cell injection tumor bioluminescence significantly increased in mice treated with CD19. CAR-Ts (from 8.6e6±2.5e6 to 1.8e8±3.9e7) but not in mice treated with CD30.28-Ts (from 5.7e6±1.5e6 to 5e7±1.8e7, $p<0.01$, n=7-9). Remarkably, we observed that CD30.CAR-Ts, while targeting CD30$^+$ EC tumor cells through the CAR (i.e. antigen-dependent targeting), also eliminated surrounding CD30$^-$ EC cells in a contact-dependent but antigen-independent manner. Specifically, we co-cultured Tera-1 cells with CD19.CAR-Ts, targeting the CD19 antigen which is not expressed by EC cells, in the presence of CD19$^-$ (K562/WT) or CD19$^+$ (K562/CD19$^+$) target cells. CD19.CAR-Ts eliminated Tera-1 cells only in the presence of K562/CD19$^+$ cells with CAR-T activation ($p<0.05$, n=6), and this effect was abrogated when CD19.CAR-Ts were separated by a transwell (n=7). We further showed that Fas/FasL interaction mediated the elimination of CD30$^-$ EC cells, which retained Fas expression. Fas knockdown in Tera-1 cells by specific siRNA rendered these cells more resistant to apoptosis when co-cultured with CD30. CAR-Ts as measured by active caspase 3 ($p<0.001$, n=4). Conversely, inducing Fas expression in the CD30$^+$ but Fas$^-$ NCCIT cells was sufficient to improve CD30.CAR-T antitumor activity ($p<0.05$, n=4). Overall, our data suggest that CD30.CAR-Ts can be used as a novel immunotherapy for ECs, as they are effective against both differentiated and stem cell-like EC cells. Additionally, we show that the Fas/FasL interaction between tumor cells and CAR-Ts can be exploited to reduce tumor escape due to heterogeneous antigen expression or to improve CAR-T antitumor activity.

RNA Virus Vectors and Small RNA Therapy

25. Transient Retroviral MS2-CRISPR/Cas9 All-in-One Particles for Efficient Targeted Gene Knockout

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The recently discovered CRISPR/Cas9 system is widely used in basic research and is an interesting tool for the treatment of genetic diseases. However, we and others showed that stable overexpression of DNA modifying enzymes leads to cytotoxicity and cells may suffer from unwanted genomic off-target events. Here, we developed transient retrovirus-based CRISPR/Cas9 all-in-one particles that co-deliver the *Streptococcus pyogenes* Cas9 (SpCas9) and the single guide RNA into target cells. Initially, we redirected the gammaretroviral (GV) packaging mechanism to generate GV MS2 bacteriophage chimera (GV.MS2) and achieved up to 70% knockout of a reporter gene in murine and human cell lines. GV.MS2-CRISPR/Cas9 all-in-one particles were dependent on Gag.MS2 structural proteins as well as the VSVg envelope protein and required co-transfection of both SpCas9 and sgRNA expression plasmids during particle production. Remarkably, knockout of the *TP53* gene in primary human foreskin fibroblasts by GV.MS2-CRISPR/Cas9 led to a growth advantage of *TP53* knockout cells versus control cells under competitive culture conditions. To analyze whether the SpCas9 enzyme is cytotoxic per se, we stably overexpressed SpCas9 in murine NIH3T3 fibroblasts at high MOIs (>10) and observed a

substantial G0/G1 cell cycle arrest associated with reduced cell growth and metabolic activity. Strikingly, these adverse side effects were absent in cultures that were treated with transient GV.MS2.SpCas9, suggesting that the observed SpCas9-mediated cytotoxicity is dose-dependent. Next, we generated alpharetrovirus-based MS2-CRISPR/Cas9 (AV.MS2-CRISPR/Cas9) all-in-one particles. Compared to GV-based CRISPR/Cas9 all-in-one particles, AV.MS2-CRISPR/Cas9 elicited similar knockout rates of our reporter gene when using up to 20-fold less supernatant. Subsequent immunoblot analysis of both chimeric particles revealed ~23-fold higher Gag.MS2 protein levels in AV.MS2-CRISPR/Cas9 supernatants, which may explain their better performance. Since chemokine receptors CXCR4 and CCR7 are promising targets for novel CRISPR/Cas9-based therapies for HIV-1 infections and/or chronic inflammatory diseases, we next tested our novel particles as potential therapeutic agents. We transduced human Jurkat cells and efficiently knocked out the endogenous CXCR4 receptor to render the cells resistant against infection with human CXCR4-tropic HIV-1-based vector particles. As further proof-of-concept, we successfully knocked out the CCR7 receptor in primary murine T cells and achieved up to 18% CCR7 negative cells. In conclusion, we introduced transient retrovirus-based CRISPR/Cas9 all-in-one particles from two different retroviral genera for efficient and non-cytotoxic, targeted gene knockout.

26. Beta-Deliverin Relieves Anti-Viral Restriction of the IFITM Proteins Thereby Enhancing VSV-G Lentiviral Vector Gene Delivery to Human Hematopoietic Stem Cells

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Lentiviral vector (LVs) gene delivery to CD34 human hematopoietic stem and progenitor cells (HSPCs) has demonstrated clinical benefit for many hematologic diseases. However, disease correction depends on efficient modification of hematopoietic stem cells (HSCs), which are resistant to LV transduction. Methods to improve HPSC transduction efficiency include increased LV dosing, repeat LV treatment, and *ex vivo* stimulation with cytokines. As these approaches face technical or expense limitations, a method to improve transduction of HSCs is desirable. Previously we reported that treatment of HSPCs with the small molecule β-deliverin improves transduction efficiency by VSV-G pseudotyped LVs 3-fold. Transduction was enhanced in umbilical cord blood and peripheral blood mobilized (mPB) HSPCs *in vitro,* through an unknown process that improved endosomal LV fusion rate. Importantly, β-deliverin treatment improved transduction and stability of GFP expression in long term repopulating cell progeny in NSG mice for 22 weeks. The clonal abundance of LV integration, with no skewing of human lineage, was interpreted to indicate preferential marking of HSCs, consistent with reports of clonal outgrowth post-transplantation. Here we report a mechanistic model of β-deliverin action. A cell line screen for β-deliverin response found upregulation of interferon response genes (ISGs), including the endosomal associated interferon-induced transmembrane proteins (IFITM2/3), reported to be expressed in HSPCs and to have anti-viral effects on HIV, VSV, and influenza. β-deliverin was found to reduce IFITM2/3 protein levels 2-3-fold in HeLa cells in a dose dependent reversible manner, as shown by western blot and subcellular analyses using confocal microscopy. IFITM2/3 vesicle number, volume and staining intensity were significantly reduced in β-deliverin treated-HeLa cells, compared to DMSO or α-deliverin (β-deliverin monomer that does not increase transduction) treated cells. β-deliverin was found to increase transduction in wild-type TZM-bl (HeLa variant) cells, but was not improved in IFITM3 knockout TZM-bl cells (the predominant IFITM expressed in HeLa cells). These findings demonstrate an anti-viral restriction role for IFITM3 and that β-deliverin treatment downregulates IFITM2/3. Finally, confocal microscopy of mPB HSPCs treated with β-deliverin demonstrated an LV-dependent phenotype for IFITM2/3-mediated restriction. Endosome activity in HSPCs was shown to be quiescent without LV administration, with IFITM2/3 vesicles localized away from the late endosome. Addition of LV was shown to activate endosomes and redistribute IFITM2/3 vesicles towards the late endosome, while β-deliverin treatment was shown to significantly reduce IFITM2/3 vesicle number (DMSO $\bar{x}$=45.08, 95% CI [39.02, 51.15] vs. β-deliverin $\bar{x}$=23.03, 95% CI [20.71, 25.34]) and IFITM2/3 intensity (DMSO $\bar{x}$=86.88, 95% CI [82.5, 91.27] vs. β-deliverin $\bar{x}$=75.95, 95% CI [71.1, 80.79]). Our findings uncover an important role for IFITM2/3 in restricting LV transduction and that β-deliverin treatment downregulates IFITM2/3, allowing enhanced transduction of HSPCs. Studies are underway to identify the role of additional ISGs in regulating LV transduction of therapeutically relevant cell types.
*Contributed Equally

27. Addressing the Impact of Vector Integration on Chromatin Architecture

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Self-Inactivating (SIN) Lentiviral Vectors (LV) have demonstrated great efficacy and safety in preclinical models and clinical trials. Still, SIN.LV integrations are not entirely neutral to the cell genome. Since genetic mutations altering chromatin architecture can lead to disease, we investigated the impact of vector integration on the host chromatin conformation. This is especially relevant for vectors carrying strong-enhancer promoters and/or chromatin insulators (CI), able to interact with distant genomic loci and whose long-range effects remain so far unexplored. To this aim, we first tested the safety of a panel of Lentiviral Vectors

(LV) harboring different human CTCF-based CI in parallel orientation, surrounding an expression cassette under the control of a strong enhancer-promoter, in an *in vivo* genotoxicity assay based on tumor prone mice (n=178 mice). Our results indicate that LV with specific CI improve the mouse survival compared to the parental LV without insulators, by reducing or abolishing vector-mediated oncogene activation. To address the underlying molecular mechanisms, we devised a LV-specific Circular Chromosome Conformation Capture (LV4C) method, able to retrieve the genomic portion flanking the LV integration site (IS) attached by proximity ligation to the corresponding host-genomic long-range interaction site (ITR). We applied LV4C to different K562 cell clones harboring multiple copies (overall >100 IS) of either the LV with a strong enhancer, which screened as genotoxic *in vivo*, or of the safer LV, harboring one of the *in vivo* tested CI. Interactions on an internal 4C-control (MYC-locus) validated our conformation-capture libraries, as highly correlating with published HiC interactions (R^2=0.9). Overall we identified >300 LV-ITR, mostly present in gene dense regions and spanning 100-500Kb from the IS. Approximately 50% of IS had multiple interaction-targets (from 2 to 11). Interestingly, we observed that the LV without CI interacts with genomic loci upstream and downstream the IS with a similar frequency. Differently, the LV with CI interacts only in one direction, upstream the IS ($p \leq 0.022$) and with genomic loci significantly enriched for CTCF-motifs in convergent orientation to the CI within the LV ($p<0.01$), the only configuration able to promote CTCF-mediated chromatin loops with insulator function. Ongoing RNA-Seq analysis will address the extent of gene deregulation at IS and ITR, to further highlight the ability of chromatin loops mediated by the CI in the LV to shield host genes from the vector enhancer. In conclusion, the CI present in the LV redirect long-range chromatin interactions towards genomic CTCF sites in convergent orientation, a mechanism we postulate to improve the safety of LV integration *in vivo*. Thus, our results promote the use of chromatin insulators to improve vector safety for future gene therapy applications. Finally, our approach could be more broadly exploited to study the impact of any regulatory element, carried by LV, on the host chromatin conformation.

28. Pharmacology and Safety of VY-HTT01, an AAV miRNA Gene Therapy Targeting Huntingtin for the Treatment of Huntington's Disease

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Huntington's disease (HD) is a fatal, inherited neurodegenerative disease that results in progressive motor, neuropsychiatric, and cognitive impairment. The disease is caused by an expanded trinucleotide repeat in the huntingtin gene (HTT), resulting in pathogenesis through a toxic gain-of-function of mutant huntingtin protein. As partial suppression of HTT in the central nervous system is both safe and effective in studies using animal models of HD, we are pursuing a therapeutic strategy focused on lowering HTT. AAV gene therapy with RNA interference (RNAi) targeting HTT mRNA selectively for degradation has the potential to provide therapeutic benefit. Here, we describe a series of in vitro and mouse experiments to identify candidate AAV gene therapies targeting HTT with RNAi, and subsequent studies in non-human primates to evaluate several candidates for HTT knockdown, examine the short-term tolerability of HTT reduction, and select a clinical candidate VY-HTT01 for the treatment of HD. All components of the AAV gene therapy targeting HTT with miRNA were optimized, including the capsid and vector genome. Initial screening was performed on approximately 120 primary microRNAs (pri-miRNAs, which include cassette and RNAi sequence) and vector genome configurations using 6 human cell lines (including HD patient fibroblasts) in vitro. Sixteen candidate AAV. miRNAs targeting HTT were identified based on knockdown and pri-miRNA processing precision and efficiency in the cell lines, and were subsequently compared in YAC128 transgenic mice expressing human mutant HTT. Based on human mutant HTT mRNA knockdown in the striatum, and precision and efficiency of pri-miRNA processing in mice, 4 lead candidates were selected for evaluation in non-human primates (*Macaca mulatta)* using similar readouts, as well as tolerability endpoints that included body weight, cageside observations, clinical pathology and histopathology. The clinical candidate, VY-HTT01, was chosen based on superior HTT lowering, high precision and efficiency of pri-miRNA processing, and safety and tolerability in the non-human primate.

29. AAV.U7-snRNA-Mediated Exon Skipping of the Toxic DUX4 Gene as a Promising Therapeutic Approach for Facioscapulohumeral Muscular Dystrophy

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Facioscapulohumeral Muscular Dystrophy (FSHD) is an autosomal dominant disorder associated with progressive muscle wasting and weakness, typically in the face, shoulder girdle, and arms. However, this pattern of muscle involvement is not universal, and some patients may develop weakness in other muscles of the body, possibly leading to wheelchair dependence and caregiver assistance. There are currently no treatments that alter the course of FSHD and therapy development is an unmet need in the field. The pathogenic mechanisms underlying FSHD have only become clear during the last decade. FSHD arises from epigenetic changes that de-repress the *DUX4* gene in muscle. The full-length isoform of DUX4 causes cell death and muscle toxicity, while a second isoform (DUX4-short; DUX4-s) is non-toxic. We hypothesize that FSHD therapies should

therefore center on inhibiting full-length *DUX4* expression. In this study, we developed a *DUX4* exon-skipping strategy designed to bias *DUX4* splicing in favor of the non-toxic DUX4-s isoform. To do this, we designed several U7-snRNAs targeting different parts of *DUX4* gene (called U7-DUX4) and demonstrated their ability to suppress full-length *DUX4* and prevent cell death *in vitro*. We then packaged our lead candidates into AAV6 particles and delivered them to muscles of our DUX4 mouse models, which develop histopathological and functional deficits associated with full-length DUX4 expression. In our first *in vivo* studies in tibialis anterior muscles injected with AAV.U7-DUX4, our lead construct ameliorated DUX4-associated histopathology, and protected muscle from damage and turnover normally caused by high levels of full-length DUX4 in mice. As of this writing, additional *in vivo* studies are underway, including testing the capabilities of our AAV.U7-DUX4 to suppress DUX4 long-term in our new mouse model, and improve histopathological, functional, and molecular outcomes. We are also performing dose-escalation experiments for efficacy and toxicology outcomes. This study provides proof-of-concept for reducing DUX4 toxicity using U7-snRNA exon skipping, and has implications for future FSHD gene therapy, both as an individual treatment and combined with AAV.RNAi targeting of *DUX4*.
Funded by the National Institutes of Health NIAMS Center of Research Translation in Muscular Dystrophy Therapeutic Development at Nationwide Children's Hospital (1P50AR070604-01).

30. Peptide Mediated Delivery of Oligonucleotides to the CNS across the Blood-Brain Barrier

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Many diseases of the central nervous system arise from the accumulation of protein such as a-synuclein in Parkinson's Disease or Aß in Alzheimer's disease. The ability to regulate the expression at the gene transcription level would be beneficial for reducing the accumulation of these proteins or regulating expression levels of other genes in the CNS. Short interfering RNA molecules can bind specifically to target RNAs and deliver them for degradation. This approach has shown promise therapeutically *in vitro* and *in vivo* in mouse models of PD and AD and other neurological disorders; however, delivery of the siRNA to the CNS *in vivo* has been achieved primarily through intra-cranial stereotaxic injection. Repeat injections by stereo-taxic injections may not be amenable to clinical translation; therefore, a new approach for delivery of siRNAs to the brain is needed. Recently, elAgnaf et al identified a small peptide from the envelope protein of the rabies virus that could deliver a siRNA via intra-venous delivery to the brain utilizing a receptor on the blood-brain barrier. However, this receptor is saturable and will only allow the delivery of a limited number of molecules. We have identified an alternative peptide for the transport of nucleotides across the BBB based on the apolipoprotein B (apoB) protein targeted to the family of low-density lipoprotein receptors (LDL-R). We used an 11-amino acid sequence from the apoB protein ($ApoB^{11}$) that, when coupled with a 9-amino acid arginine linker, can transport siRNAs across the BBB to neuronal and glial cells. To examine the effectiveness of this peptide mediated oligonucleotide delivery system, we delivered a siRNA targeted to the α-synuclein gene in a mouse model of Parkinson's disease. We found α-syn siRNA co-localized to neurons and glial cells and subsequent reduction in α-synuclein protein accumulation in the neurons of the mouse across the whole brain. Furthermore, we observed increased neuronal numbers and decreased astrogliosis following delivery of the $ApoB^{11}$-si-αsyn compared to controls. Thus, we have identified an alternative delivery route for siRNA molecules targeted an alternative receptor allowing the delivery of multiple, simultaneous therapeutics.

Hematopoietic Cell Therapies

31. A Novel Target for Hematopoietic Stem Cell (HSC) Gene Therapy and Editing

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There are now many hematological diseases and disorders that can be successfully treated with hematopoietic stem cell (HSC) gene therapy. Most if not all of these approaches target $CD34^+$ cell fractions. However, $CD34^+$ cells are a heterogeneous mix of mostly progenitor cells and only very few long-term engrafting HSCs. Despite several decades of research, there is still considerable debate about the phenotypical and transcriptional identity of "true" HSCs. Available treatment strategies would greatly benefit from the ability to isolate, target and modify a more enriched hematopoietic stem/progenitor cell subset that provides short-term reconstitution as well as long-term multilineage engraftment. Performing competitive repopulation experiments of CD34 subpopulations in the pre-clinical nonhuman primate (NHP) model, we have recently identified a novel HSC-enriched phenotype ($CD90^+CD45RA^-$). This HSC-enriched phenotype was exclusively responsible for rapid short-term recovery, robust multilineage engraftment in the peripheral blood (PB) as well as entire reconstitution of the bone marrow (BM) stem cell compartment. To further characterize and evaluate this phenotype for potential clinical applications, we performed comprehensive transplantation studies with equivalent human CD34 subpopulations in mice, analyzed the heterogeneity by single cell RNA sequencing (scRNAseq) and finally compared the phenotypical as well as transcriptional composition of $CD90^+CD45RA^-$ HSPCs to alternative HSC-enrichment strategies. Similar to our NHP studies, human multilineage engraftment in the PB, BM, spleen and thymus of sublethally irradiated adult NSG mice was nearly entirely restricted to freshly-isolated and sort-purified $CD90^+CD45RA^-$ cell fractions. Most importantly, engraftment of phenotypical and functional human HSCs as well as reconstitution of downstream progenitors in the BM stem cell compartment was exclusively observed for mice transplanted with $CD90^+CD45RA^-$ HSPCs. To further characterize $CD90^+CD45RA^-$ HSPCs, we

next performed scRNAseq and determined the transcriptional heterogeneity. In comparison to gold standard CD34$^+$ cells, CD90$^+$CD45RA$^-$ HSPCs were nearly entirely depleted for lineage-committed progenitor cells, whereas transcriptionally distinct clusters containing multipotent progenitors (MPPs) and HSCs were significantly enriched. Finally, we compared the phenotypical and transcriptional heterogeneity of CD90$^+$CD45RA$^-$ HSPCs to alternative HSC-enrichment strategies including CD133$^+$ and CD34$^+$CD38$^{low/-}$ cell fractions. In this side-by-side comparison, CD90$^+$CD45RA$^-$ cell fractions demonstrated the highest enrichment for phenotypically, functionally as well as transcriptionally primitive HSCs/MPPs. Thus, we conclude that CD90$^+$CD45RA$^-$ HSPCs demonstrate the most refined and enriched target population for HSC gene therapy and editing. Purification of this HSC-enriched phenotype has the potential to decrease (20 to 30-fold) the number of target cells, reduce the costs of editing reagents and improve the overall feasibility of currently available approaches.

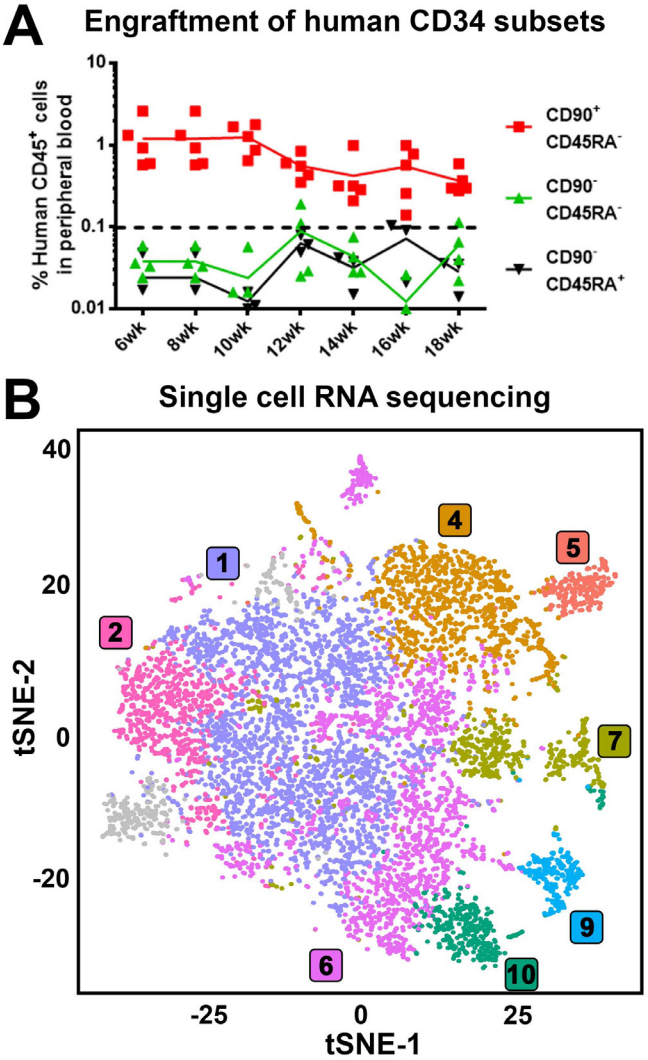

Figure legend: A) Engraftment of human cells in the PB of mice transplanted with human CD34 subpopulations. B) Graph-based clustering of CD34 subsets.

32. Immunotoxin-Based Conditioning Facilitates Autologous Hematopoietic Stem Cell Engraftment and Multi-Lineage Development in a Fanconi Anemia Mouse Model

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Hematopoietic stem cell (HSC) gene therapy is a promising approach for the treatment of inherited bone marrow failure disorders such as Fanconi Anemia (FA). Conditioning chemotherapy is used to deplete hematopoietic stem cells in the recipient's marrow, facilitating engraftment of donor cells. While effective, some major issues with chemotherapy include genotoxic effects increasing the risk of secondary malignancies. Antibody conjugates targeting hematopoietic cells is an emerging non-genotoxic method of opening the marrow niche and promoting engraftment of transplanted cells. Prior studies have shown that CD45 or CD117 monoclonal antibodies facilitate donor engraftment in mice. This platform would be ideal in diseases such as FA which is associated with sensitivity to DNA damage and cancer predisposition. Avoiding alkylating agents could improve outcomes and success rates in these patients. Furthermore, this methodology could be translated to the allogeneic bone marrow transplantation setting, decreasing the toxicity of this treatment modality. Our approach utilizes immunotoxin conjugates as an alternative conditioning regimen in an FA mouse model of autologous transplantation. Antibodies targeting either CD45 or CD117 epitopes were conjugated to saporin (SAP), a ribosomal toxin. Mice carrying a knockout of the *FancA* gene were conditioned with either CD45-SAP or CD117-SAP prior to receiving whole marrow from a heterozygous healthy donor. On the day of transplantation, mice conditioned with immunotoxin exhibited equivalent levels of LSK depletion in the bone marrow and corresponding lack of CFU potential, similar to cyclophosphamide treated controls. Immunotoxin-treated groups initially exhibited lower levels of peripheral donor engraftment, but there was no significant difference between CD45-SAP and cyclophosphamide groups at one-month post-transplant. By three months post-transplant, both immunotoxin-treated groups displayed similar engraftment levels as cyclophosphamide treated controls (Figure 1). Bone marrow chimerisms at six months also showed equivalent levels of engrafted LSK stem cell populations between immunotoxin- and cyclophosphamide-treated groups (Figure 2). Furthermore, cyclophosphamide treated mice had greater and sustained weight loss than our immunotoxin groups. Our findings suggest immunotoxins may be an effective conditioning strategy in HSPC transplantation, especially in diseases where traditional chemotherapy is not tolerated. We now are initiating studies using immunotoxin-based conditioning for the transplantation of gene-modified syngeneic stem cells and allogeneic cells. We think these studies will inform future clinical trials and provide the groundwork for the next-generation of therapy for FA patients.

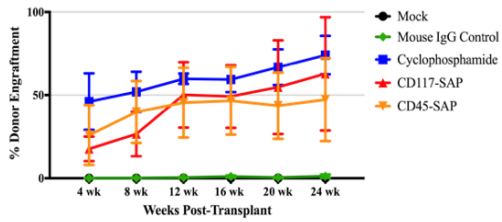

Figure 1. Percent donor engraftment as estimated by GFP expression in total peripheral blood

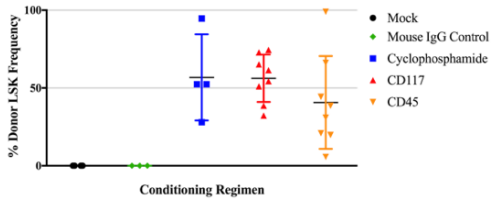

Figure 2. Percent donor-derived stem cells in the bone marrow at 24 weeks post-transplant as estimated by GFP expression in LSK cells (Lineage$^-$Sca$^+$c-Kit$^+$)

33. Development of a New Generation of Gene-Edited HSC to Induce Engraftment Advantage and Favour Outgrowth In Vivo

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Hematopoietic stem and progenitor cells gene therapy (HSPC-GT) with gene transfer vectors has successfully entered clinical testing for several types of inherited diseases of the immune, hematopoietic system and some storage diseases. More recently, gene editing (GE) is being investigated to provide a more precise gene correction strategy. However, autologous HSPC-GT and GE still require myeloablative preconditioning treatment with genotoxic drugs that may have substantial long-term effects. Moreover, In the case of GE, the yield of edited HSPC remains limited and their long-term engraftment capacity to be demonstrated. Thus, there is a need to develop improved strategies supporting engraftment of the gene modified HSPC. We investigated, taking advantage of genetic tools, the modulation of two different target genes, expressed on the HSPC membrane and involved in phagocytosis protection and homing, respectively. As a first attempt, we stably upregulated expression of these two genes. In in vivo assays, after transplantation into NSG mice, the genetic modification of HSPC increased human cell engraftment at long term, indicating preferential benefit to the more primitive HSPC. Ongoing studies are investigating the best window of time and transient upregulation strategies to exploit these benefits. We are now investigating mRNA delivery to transiently induce a gain-of-function in the edited cells, in order to promote their outgrowth in vivo. In parallel, we established an experimental model to study human HSPC mobilization from the bone marrow niche and tested modified HSPC advantage in niche re-colonization. NSG mice stably engrafted with human HSPC were treated for mobilization, then infused with gene marked control or genetically modified HSPC from the same donor as the original transplant. Remarkably, advantaged HSPC efficiently outcompeted the mobilized HSPC and established stable chimerism at ≥10% in the human cell graft, while control cells were only detectable at 1-2 % level. We are now working towards further increasing the efficacy of engraftment of gene corrected cells using transient O/E. If successful, it may be eventually possible to reach chimerism levels with gene corrected cells in the autologous setting that may be sufficient to provide long-term benefit in some diseases, while at the same time sparing patients toxic preconditioning regimens.

34. Towards a Mechanistic Understanding of Ex Vivo Hematopoietic Stem Cell Expansion for Gene Therapy

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Ex vivo expansion of human hematopoietic stem and progenitor cells (HSPC) is a long-sought goal for gene therapy, with the potential to alleviate delayed hematopoietic reconstitution after myeloablation and enable clinical application of emerging technologies such as antibody-based conditioning and gene editing. Several compounds facilitated successful expansion of CB CD34+ cells in clinical trials, but it remains to be determined how they compare to each other and whether the respective protocols can be transferred to genetically engineered HSPC from mobilized peripheral blood (MPB) or BM. Simple assays to compare different compounds and protocols are direly needed to optimize gene therapy-tailored expansion protocols. Using a protocol based on the AhR antagonist SR1, we have previously shown that engrafting cells can be prospectively assigned to the CD34+CD90+ cell compartment upon 7-14 days in culture. Addition of UM171 substantially increased the number of CD34+CD90+ cells and upregulated EPCR on a fraction of these cells in a dose-dependent manner. In our hands, a 7 day culture in UM171 alone expanded SCID repopulating cells from lentivirally transduced MPB 3-4 fold compared to cells transduced with a short 36hr ex vivo protocol. To confirm the immunophenotypic compartment where engraftment potential resided, we sorted MPB CD34+ cells into 2-4 fractions based on CD90 and EPCR expression after 36hr or 7 days in UM171 culture, transduced them with lentiviral vectors coding for different fluorescent proteins and mixed the individually marked fractions to track their contributions to NSG repopulation. Only 3% of CD34+ cells were EPCR+CD90+ at 36hr, but this fraction increased during culture peaking around day 7. CD34+CD90+EPCR+ cells stably contributed up to 70% of multi-lineage short- and long-term reconstitution in NSG mice. We conclude that the CD34+90+EPCR+ phenotype can serve as a reliable HSC marker for lentivirally-transduced MPB cells, and a brief UM171 pulse followed by sorting of CD34+CD90+EPCR+ cells may be experimentally and clinically exploited to enrich cultured MPB for engrafting HSPC. EPCR was particularly useful during the first days of culture, where it marked only about 10% of CD34+CD90+ MPB cells, while it converged with the CD34+CD90+ phenotype during late time-points. To capture the biology of expanding HSC and identify novel markers facilitating their prospective isolation, we performed serial single cell RNA sequencing (scRNAseq) on the Chromium 10x platform to resolve population heterogeneity during UM171 expansion culture of MPB CD34+ cells. tSNE analysis identified 2 macroclusters: surprisingly, the principal cluster comprising 73% and 78% of the cells on days 3 and 7 of culture, respectively, was characterized by expression of erythroid/megakaryocyte genes, while the minor cluster

(27% and 22%) predominantly expressed myeloid progenitor genes. Importantly, engrafting CD90+EPCR+ cells mapped to the latter cluster. Differentially expressed genes mapped to ROS metabolism and HOX genes. ScRNAseq represents a powerful technology to gain mechanistic insight into ex vivo HSC expansion. Comparative scRNAseq studies between different expansion principles are ongoing and will help identifying an optimal HSC expansion protocol for gene therapy applications.

35. Long-Term Human Protein Expression in Mice via Engraftment of Gene-Edited Human Plasma Cells

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Activated B cells have the ability to differentiate into antibody-secreting, long-lived plasma cells, which provide long-term humoral immunity. Because of their high secretory capacity and longevity, long-lived plasma cells are an attractive candidate for cell-based protein replacement therapy. By simultaneously delivering CRISPR/Cas9 ribonucleoprotein complexes and recombinant adeno-associated viruses carrying homologous DNA donor templates, we achieved specific integration of exogenous gene cassettes in primary human B cells at rates of 20-40%. Edited cells were subsequently differentiated into plasma cells *in vitro*. Using this strategy, we generated plasma cells that secreted functional factor IX, the coagulation factor implicated in hemophilia B, with a specific activity of 63 IU/mg. Further, plasma cells engineered to secrete an exogenous human cytokine, B-cell activating factor (human BAFF; a potent B cell survival factor), exhibited increased survival and differentiation *in vitro*. Here, we evaluated the *in vivo* engraftment capacity of gene edited human plasma cells and the *in vitro* survival and function of edited plasma cells in long-term cultures. We show that gene edited plasma cells engrafted in immunodeficient mice and secreted high levels of human antibodies *in vivo*. Further, recipients of plasma cells programmed to secrete BAFF had substantial levels of this protein in their peripheral circulation. Mice that received BAFF-expressing plasma cells also had significantly higher serum antibody levels, demonstrating a possible strategy for improving plasma cell engraftment. Importantly, secreted human proteins were detected for months in the serum of recipient mice, indicating that a subset of engrafted plasma cells may be long-lived. We will also present the results of ongoing studies assessing the effects of additional key survival factors, such as interleukin 6 (IL6) and a proliferation-inducing ligand (APRIL), on plasma cell survival and secretion *in vivo* using novel humanized mouse models. Finally, we found that plasma cells could be sustained in culture for at least one month following their *in vitro* differentiation by supplementing the cultures with cytokines, including IL6, interferon alpha and/or APRIL. These cells maintained high levels of antibody production *in vitro*, demonstrating that they retained their secretory functions in long-term cultures. Together, these studies highlight the potential of using gene-edited plasma cells as "protein factories" to treat protein deficiencies, such as hemophilia.

773. Macrophage Polarization Impacts Tunneling Nanotube Formation and Organelle Trafficking Following Hematopoietic Stem Cell Therapy for Cystinosis

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Tunneling nanotubes (TNTs) are a recently discovered method of long range intercellular communication. They enable cytosol-to-cytosol connections and facilitate trafficking of cargo ranging from small molecules to organelles between numerous cell types both *in vitro* and *in vivo*. In the decade since discovery, TNTs have been implicated in biological functions ranging from pathogen spreading to cancer resistance, and our research indicates that they provide a novel therapeutic route for delivering engineered gene products across the body. We have observed TNT formation from macrophages following hematopoietic stem and progenitor cell (HSPC) transplantation therapy for cystinosis, a genetic lysosomal storage disorder wherein cystine accumulates in the lysosome and causes tissue degeneration and multi-organ failure. We previously showed in *Ctns*$^{-/-}$ mice that a single HSPC treatment causes life-long preservation of tissue morphology and function. This occurs via transport of cystinosin-bearing lysosomes from HSPC-derived macrophages to diseased cells through TNTs. These findings demonstrate a central role of macrophages in cell-based regenerative therapies by their capacity of generating TNTs that can deliver functional organelles to diseased tissue. We now seek to more precisely understand the phenotype of HSPC-derived macrophages in regards to TNT-mediated tissue repair. Macrophages have broad functions ranging from phagocytic and pro-inflammatory to homeostatic and anti-inflammatory. We polarized bone marrow derived macrophages (BMDMs) expressing cystinosin-eGFP to either a pro- or anti-inflammatory phenotype and then developed novel imaging analysis programs to quantify TNT formation and intercellular trafficking to diseased fibroblasts following co-culture. We discovered that fewer TNTs formed with less transfer of cystinosin-eGFP in the pro-inflammatory condition vs. anti-inflammatory or control. To extend these findings *in vivo,* BMDMs were isolated from two transgenic mice strains deficient in anti-inflammatory macrophage polarization (MAFIA and Rac2$^{-/-}$). To our surprise, *Ctns*$^{-/-}$ mice responded equally well to transplantation of mutant and wildtype HSPCs. Furthermore, there was no difference in TNT formation or organelle trafficking *in vitro*. Polarized macrophages are dynamically flexible and best considered an activity spectrum rather than fixed dichotomy, but cytokine treatment *in vitro* masks much of this complexity by pushing the phenotype to non-physiological extremes. Our data therefore highlight how *in vivo* models are essential to understand physiological macrophage behavior. In conclusion, these findings reveal heretofore unrealized subtleties in the cellular mechanisms of TNT formation and function. This mechanistic understanding of stem cell transplantation may facilitate novel applications for both cystinosis therapy as well as other regenerative medicine approaches.

36. Abstract Withdrawn

Advances in CRISPR/Cas Technologies

37. Development of Orthogonal Cas9-Cas9 Fusion Proteins and Their Potential Application as β-Hemoglobinopathy Therapeutics

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The development of robust, versatile, and accurate toolsets is critical for the advancement of therapeutic genome editing applications. We recently reported a chimeric fusion of an attenuated *S. pyogenes* Cas9 nuclease (SpyCas9^{MT}) to a DNA-binding-domain (DBD) that displays enhanced targeting range and improved specificity[1]. Here we extend this platform to include Cas9-Cas9 chimeras by replacing the DBD with an orthogonal Cas9 from *N. meningiditis* (NmeCas9) or *S. aureus* (SauCas9), which produces a system that is entirely RNA-programmable. This Cas9-Cas9 system can be used in both single- and dual-nuclease formats, which establishes a versatile genome engineering platform. In the single nuclease format, SpyCas9 is fused to nuclease dead NmeCas9 or SauCas9. The nuclease activity of attenuated SpyCas9^{MT} is restored when delivered to its target site as a SpyCas9^{MT}-dNme/SauCas9 fusion due to its increased effective concentration. GUIDE-seq analysis and targeted deep sequencing of potential off-target sites indicates that the SpyCas9^{MT}-dNme/SauCas9 fusions display excellent accuracy. In the dual nuclease format, where both Cas9 nucleases are active, we find that synchronous cleavage of the target genome at two neighboring positions produces primarily precise segmental deletions (as high as 97% of all lesions). Analysis of more than 40 genomic target sites revealed that the total level of editing and the fraction of precise segmental deletions are higher for Cas9-Cas9 nucleases than a pair of independent Cas9 monomers (median rate ~90%). Thus, dual nuclease Cas9-Cas9 fusions produce predominantly defined sequence products within the edited genome. For the *ex vivo* disruption of therapeutically relevant genes or regulatory elements, we generated Cas9-Cas9 fusion proteins by utilizing split intein-mediated protein *trans*-splicing. We demonstrate that Cas9-Cas9 ribonucleoproteins (RNPs) generated with synthetic guide RNAs have robust activity when electroporated into human cells. These RNPs can efficiently delete transcriptional regulatory elements, such as the erythroid enhancer of *BCL11A,* within the genome of CD34+ hematopoietic stem and progenitor cells resulting in upregulation of gamma-globin expression in differentiated erythroblasts. GUIDE-seq and targeted deep-sequencing analysis indicate that there are few active off-target sites within the genome for many of the programmed dual nucleases, and that some of the observed off-target activity can be effectively suppressed by utilizing attenuated-SpyCas9 in the context of the Cas9-Cas9 fusion proteins. Thus Cas9-Cas9 chimeras represent an important new tool that could be particularly valuable for therapeutic genome editing applications, such as hematopoietic stem cell treatments for beta-hemoglobinopathies, where precise genomic cleavage and defined end products are desirable.

1.Bolukbasi, M.F. et al. DNA-binding-domain fusions enhance the targeting range and precision of Cas9. *Nat Methods* **12**, 1150-1156 (2015).

38. RNA Secondary Structure Increases the Specificity of Class 2 CRISPR Effectors

D Dewran Kocak, Eric A. Josephs, Jennifer B. Kwon, Shaunak S. Adkar, Charles A. Gersbach

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CRISPR systems have been broadly adopted for basic science and biotechnology applications, and are being widely explored for gene and cell therapy. In some cases, these bacterial nucleases have demonstrated off-target activity when applied in mammalian cells. This creates a potential hazard for therapeutic applications and could confound results in biological research. Therefore, improving the specificity of these nucleases is of broad interest. Here we show that engineering a hairpin secondary structure onto the spacer region of the guide RNA (hp-gRNAs) can increase specificity by several orders of magnitude when combined with various CRISPR effectors. Specifically, we engineered RNA secondary structure onto the spacer by extending a designed hairpin on the 5' end of the gRNA (hp-gRNA). The resulting hairpin structure then serves as a steric barrier to strand invasion. We expected that by adjusting the strength of the secondary structure, strand-invasion could proceed to completion at the on-target site, but could be impeded at off-target sites, which often have reduced energetics due to RNA-DNA mispairing. Since strand invasion of the target sequence by the gRNA is the critical process governing SpCas9 nuclease activity, this would block off-target nuclease activity and result in an increase in specificity. Focusing first on Cas9 from *S. pyogenes* (SpCas9), we show that rationally designed hp-gRNAs can tune SpCas9 binding to genomic DNA. We then show that hp-gRNAs increase the specificity of gene editing using five different Cas9 or Cpf1 variants. Though these effectors have diverse structures and different mechanisms of target recognition, we observe that hp-gRNAs function similarly across effectors, inducing decreases in nuclease activity proportional to the strength of the hp-gRNAs engineered secondary structure. Importantly, these decreases occur preferentially at off-target loci. To gain insight into potential mechanisms of specificity increases driven by hp-gRNAs, we use atomic force microscopy to measure both binding and structure of various Cas effectors at both on-target and off-target sites. Our results demonstrate that RNA secondary structure is a fundamental parameter that can tune the activity of class 2 CRISPR systems.

39. An Enhanced CRISPR-Cas12a Variant to Improve Genome and Epigenome Editing Efficacy

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CRISPR-Cas12a genome editing nucleases, formerly known as Cpf1, possess various distinct properties relative to the commonly used *Streptococcus pyogenes* Cas9 (SpCas9), including: recognition of an extended T-rich PAM, catalysis that generates 5'-overhangs, the ability to process individual crRNAs out of a single transcript, and the requirement for only a single short ~40 nt crRNA[1]. We have previously demonstrated that two Cas12a orthologues from *Acidaminococcus sp. BV3L6* and *Lachnospiraceae bacterium ND2006* (AsCas12a and LbCas12a, respectively) can robustly function for genome editing in a variety of human cell types and that they possess high genome-wide specificities[2]. However, both AsCas12a and LbCas12a recognize a PAM of the form TTTV[1] (where V is any nucleotide other than T), substantially limiting targeting relative to the NGG PAM motif utilized by SpCas9. To improve the targeting range of CRISPR-Cas12a nucleases, we utilized AsCas12a and LbCas12a crystal structures to perform rational mutagenesis of residues that lie within 10 angstroms of the PAM nucleotides. We generated Cas12a variants harboring various combinations of mutations at these positions and characterized their on-target activities and genome-wide specificities in human cells. These engineered Cas12a PAM variants have greatly improved activity compared to their wild-type counterparts on canonical TTTV and TTTT PAM sequences, and importantly, also show robust activity on sites with PAMs of the form NTTV, TATV, TTCV, and other PAM sequences that are not efficiently cleaved by wild-type Cas12a. These variants expand PAM recognition from TTTV to a variant of the NWYV motif (where W is A or T, and Y is T or C), increasing the targeting range by more than six-fold. Preliminary GUIDE-seq analyses suggest that these engineered Cas12a variants exhibit only a modest difference in specificity compared to wild-type Cas12a, and that supplementary mutations analogous to those present in our previously described high-fidelity SpCas9 (SpCas9-HF) variants[3] can be implemented to retain high genome-wide specificities for engineered Cas12a nucleases. The improved on-target activities and expanded targeting range of the Cas12a variant are also demonstrated in the context of epigenome editing constructs, where the catalytically inactivated dCas12a is fused to the synthetic VPR activator. We will present data that demonstrates potent gene activation at levels greater than published LbCas12a-VPR fusions[4], and that these epigenome editing constructs now have the benefit of a greatly expanded targeting range for more fine-tuned non-coding element mapping. Together, our results demonstrate that both natural and engineered forms of CRISPR-Cas12a nucleases are robust and useful enzymes, and that our Cas12a PAM variants expand targeting range and improve both genome and epigenome editing activities. These findings should encourage broader implementation of engineered Cas12a reagents for both research and therapeutic applications.

References: 1) Zetsche & Gootenberg et al. (2015) *Cell* 2) Kleinstiver & Tsai et al. (2016) *Nature Biotechnology* 3) Kleinstiver & Pattanayak et al. (2016) *Nature* 4) Tak et al. (2017) *Nature Methods*

40. Multiplex Human T Cell Engineering by Cas9 Base Editor Technology

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Chimeric antigen-receptor (CAR) engineered T cells have mediated impressive outcomes in a subset of hematological malignancies, yet this therapy remains highly personalized and largely ineffective against more widespread epithelial and solid tumors. Cellular manufacturing processes tailored to individual patients, and an inability to overcome the complex and immunosuppressive properties of the solid tumor microenvironment remain formidable challenges in the effort to achieve widespread application of adoptive cell therapies for cancer. Genome editing strategies using targeted nuclease platforms are being developed to overcome these limitations, and several are already entering clinical application. Multiplex gene editing strategies to develop off-the-shelf cellular therapies against non-hematological malignancies are of particularly high interest but remain limited by concerns surrounding off-target effects and chromosomal translocations induced by simultaneous double-strand break (DSB) induction at multiple genomic loci. The risk of genotoxic side-effects is further amplified when combining multiplex DSB induction with randomly integrating platforms for antigen-specific receptor delivery. An ideal strategy would allow for multi-gene disruption and targeted integration of antigen-specific receptors without introducing multiple genomic DSB. To this end, we evaluated the application of third- and fourth-generation Cas9 base-editor technologies for gene disruption in primary human T cells. Through systematic reagent and dose optimization efforts we achieved highly efficient C > T base conversion and consequent protein knockout at multiple therapeutically relevant loci including TRAC (KO = 80.8%, SD = 10.7%), PD1 (79%, SD = 4%), and B2M (80%, SD = 3%). We observed that fourth generation base editor (BE4) achieved consistently higher C > T conversion rates with reduced non-canonical editing (i.e. C>A, G) overall compared to third generation (BE3). Targeted disruption of splice acceptor (SA) and splice donor (SD) sites resulted in higher frequency of protein knockout versus induction of premature stop codons at all loci examined. Importantly, while multiplex editing of these loci using Cas9 nuclease resulted in detectable translocations between all targeted sites, we were unable to detect these translocations using BE4 as measured by PCR. Finally, we exploited the single strand nickase function of BE4 in conjunction with rAAV delivery to achieve simultaneous targeted integration of a gene expression cassette at the AAVS1 safe harbor locus at rates exceeding 70%. Collectively, we demonstrate that Cas9 base-editor technology can be utilized to mediate efficient, multiplex gene disruption and targeted gene integration in primary human T cells without associated translocations. This streamlined approach to genome engineering may be broadly applied for the development of safe and effective cell therapies.

41. Efficient Genome Editing in Primary Human T, B and HSCs Using Baboon Envelope Gp Pseudotyped Viral Derived "Nanoblades" Loaded with Cas9/sgRNA Ribonucleoproteins

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Programmable nucleases have enabled rapid and accessible genome engineering in eukaryotic cells and living organisms. However, their delivery into target cells can be technically challenging when working with primary cells. Here, we have designed "Nanoblades", a new technology that will deliver a genomic cleaving agent into cells. These are genetically modified Murine Leukemia Virus (MLV) or HIV derived virus like particle (VLP), in which the viral structural protein Gag has been fused to the Cas9. These VLPs are thus loaded with Cas9 protein together with the guide RNAs. Thus Nanoblades are devoid of any viral-derived genetic material. Nanoblades are extremely efficient in entry and delivery of their Cas9/sgRNA ribonucleoproteins cargo into murin and human cell lines when pseudotyped with VSV-G envelope. Additionally, they are remarkably efficient for entry into human T, B and hematopoietic stem cells thanks to their surface co-pseudotyping with baboon retroviral and VSVG envelope glycoproteins. We were able to induce efficient, transient and very rapidly genome-editing in human induced pluripotent stem cells reaching up to 70% in the empty spiracles homeobox 1 (EMX1) and muscular dystrophy (MD) gene locus. A brief nanoblade incubation with primary human T and B cells resulted in 40% and 20% editing of the Wiskott-Aldrich syndrome (WAS) gene locus, respectively, while hematopoietic stem cells treated for 18 h with nanoblades allowed 30-40% gene editing in the WAS gene locus and up to 80% for the Myd88 genomic target. Moreover, no cell toxicity and low to undetectable off-target effects for as well the HIV- and MLV-derived nanoblades were demonstrated. Additionally, nanoblades can be complexed with donor DNA for "all-in-one" homology-directed repair' in order to correct a gene or programmed with modified Cas9 variants to mediate transcriptional up-regulation of target genes. Summarizing, this new technology is simple to implement in any laboratory, shows high flexibility for different targets including primary immune cells of murine and human origin, is relatively inexpensive and therefore have important prospects for basic and clinical translation in the area of gene therapy.

42. *In Vivo* CRISPR-Cas Genome Editing with No Detectable Off-Target Mutations

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CRISPR-Cas genome-editing nucleases hold substantial promise for human therapeutics but defining frequencies of unwanted off-target mutations remains an important requirement for clinical translation. Here we demonstrate that CRISPR-Cas nucleases with appropriately designed guide RNAs (gRNAs) can induce efficient *in vivo* editing in mice without inducing detectable off-target mutations as judged by a highly sensitive and generalizable strategy called VIVO (Verification of In Vivo Off-targets; see accompanying abstract by Akcakaya et al.). For this study, we sought to characterize *in vivo* genome-wide off-target mutation profiles of SpCas9 gRNAs designed to be relatively orthogonal to the mouse genome. We constructed two gRNAs, named gM and gMH, to target coding sequence sites in the mouse *Pcsk9* gene. These were chosen because of relatively few closely matched sites (sites with one, two or three mismatches relative to intended on-target sites) in the C57BL6/N mouse genome. We delivered each gRNA with SpCas9 nuclease to mouse liver using adenoviral vectors and identified efficient and stable modification of on-target mouse *Pcsk9* site with both gRNAs in mouse liver. Additionally, we observed significant stable reductions of mouse Pcsk9 protein in plasma with gM and gMH. We used VIVO to identify potential off-target mutations for gM and gMH. VIVO consists of two steps: (1) an initial *in vitro* "discovery" step using CIRCLE-seq to identify potential off-target cleavage sites for a nuclease of interest on purified genomic DNA; and (2) a subsequent *in vivo* "confirmation" step examining targeted amplicon sequencing of potential off-target sites identified by CIRCLE-seq for evidence of indel mutations *in vivo*. CIRCLE-seq experiments performed with SpCas9 and gM or gMH identified hundreds of potential off-target sites, with most having three or more mismatches relative to the on-target site. For gM, we used targeted amplicon sequencing to examine the on-target site and all 181 off-target cleavage sites identified *in vitro* by CIRCLE-seq. Strikingly, the only site that showed significant indel mutations (relative to mice treated with the control SpCas9/GFP virus) was the intended gM on-target site with indel frequencies ranging from 12.6-18.5%. For gMH, because CIRCLE-seq identified a large number of potential off-target sites (529 total), we examined the on-target site and a subset of 62 potential off-target sites with up to six mismatches that had the highest CIRCLE-seq read counts. These sites encompassed all of the CIRCLE-seq sites that had up to three mismatches. Our choice to test

this subset of sites for the gMH gRNA was guided by our findings that sites with four or more mismatches were not mutated by gP/SpCas9 *in vivo*. Among these 63 sites, we found significant indel mutations at only the on-target mouse gMH site (indel frequencies ranged from 27.4-43.6%). Our studies show that appropriately designed gRNAs can direct efficient *in vivo* editing in mouse liver without inducing detectable off-target mutations as judged by VIVO. Taken together, these assays and results provide important methodologies and strong support for the development of *in vivo* genome editing therapeutic strategies.

43. Enhanced Genome Editing Using Split *Staphylococcus Aureus* Cas9 Delivered in Double-Stranded Adeno-Associated Viral Vectors

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The possibility to easily and specifically edit the mammalian genome using the RNA-guided bacterial Cas9 endonuclease bears enormous potential for gene therapy. Particularly attractive is that Cas9 and guide RNA, the two sole components needed for gene editing, can be encoded and delivered to target cells by viral vectors. To this end, most promising candidates are Adeno-associated viruses (AAVs) due to their apathogenicity and their great performance *ex vivo* or *in vivo*, governed by the serotype origin of the capsid. Still, a limitation of this vector is the single-stranded (ss) nature of the AAV DNA genome, which necessitates a slow and rate-limiting conversion to expression-competent double-stranded vector DNA in the transduced cell. This restriction can be overcome by mutating the inverted terminal repeats at the end of the genome, yielding "double-stranded" (ds)AAV genomes that self-anneal in the transduced cells and hence express their transgenes more rapidly and more efficiently than conventional AAV vectors. Yet, this comes at the cost of a further reduction of the AAV packaging capacity, from about 5 kb for traditional ssAAVs, to only 2.4 kb for dsAAVs. Previously, others have circumvented the inherent size limit of AAV vectors by splitting the *Streptococcus pyogenes* (*Sp*)Cas9 endonuclease into two parts which are reconstituted in the cell to the full-length protein via chemically inducible dimerizers or by harnessing intein *trans*-splicing. For the latter, the N- and C-terminal parts of split inteins are fused to each protein half. Upon assembly, the inteins autocatalytically splice themselves out of the protein and connect their flanking sequences through a newly synthesized peptide bond. Here, we adapted the intein *trans*-splicing mechanism to the Cas9 sequence from *Staphylococcus aureus* (*Sa*Cas9), which is smaller than *Sp*Cas9 (3.1 versus 4.2 kilobases) and potentially more specific due to more stringent requirements for target DNA recognition. Accordingly, we split *Sa*Cas9 into two individual halves of roughly equal sizes, each of which was sufficiently small to allow packaging as dsAAV vectors. We then used these to target the endogenous mecp2 locus in the Neuro 2A cell line and in primary myoblasts, as well as the coagulation factor IX in murine primary hepatocytes. Notably, the new split dsAAV-*Sa*Cas9 vectors gave up to six times higher knock-out efficiencies compared to the common full-length *Sa*Cas9 in ssAAV, depending on cell type. Moreover, formation of on-target insertions/deletions was observed already one day after transduction of split *Sa*Cas9 and thus more rapidly than with the conventional full-length nuclease. In our study, we provide the first proof-of-concept that the original combination of dsAAV, intein *trans*-splicing and *Sa*Cas9 has great potential to further improve the efficiency and kinetics of gene editing in mammalian cells. Thereby, our new vectors constitute a new and most promising addition to the current CRISPR/Cas toolkit that expands the range of basic and therapeutic applications of genome engineering.

Hematologic & Immunologic Diseases II

44. Selective HSC-Ablation Using CD117 Antibody-Drug-Conjugates Enables Safe and Effective Murine and Human Hematopoietic Stem Cell Transplantation

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Hematopoietic stem cell transplantation (HSCT) has the potential to cure virtually any genetic blood or immune disease, and can be used to treat a variety of other conditions. However, despite its potential, HSCT is used in <25% of patients who could benefit. This is primarily due to high morbidity/mortality from associated graft versus host disease (GvHD) and the toxicities from irradiation/chemotherapy conditioning that are currently employed to enable donor HSC engraftment. Autologous gene-therapy eliminates GvHD risk, however genotoxic conditioning is still required to enable engraftment of gene modified HSCs. Although many efforts have been aimed at reducing these conditioning agents, even low doses can cause debilitating side effects including mucositis, multi-organ damage, infertility, secondary malignancies, and cytopenias which can lead to deadly infections. Eliminating genotoxic conditioning entirely with preservation of the immune system would dramatically improve HSCT, which would be especially powerful when combined with gene-therapy/gene-editing thereby reducing all problems with transplantation today. We have previously shown that competition with host HSC limits donor HSC engraftment, and that antagonistic anti-CD117 antibodies depleting host HSC are an effective, safe alternative conditioning approach in immunodeficient mice. However, to enable use in wild-type mice, this approach needed to be combined with other agents making clinical translation difficult. We subsequently showed non-genotoxic CD45-immunotoxins avoid these concerns and enable high levels of donor chimerism in wildtype mice, however they cause a transient immune depletion as CD45 is also present on lymphocytes. To overcome these challenges, we generated CD117 antibody-drug-conjugates by linking

anti-CD117 antibodies to the protein synthesis toxin saporin. Here we show that these anti-mouse CD117-ADCs cause >99.9% depletion of host HSCs in wildtype mice and subsequently enable rapid >99.9±0.1% engraftment of donor whole bone marrow cells and >69.0±12.8% engraftment of more difficult to transplant donor purified HSCs. This regimen was found to uniquely spare the peripheral blood. It does not cause significant cytopenias requiring transfusions and unlike classical conditioning regimens, does not cause any neutropenia. The immune system of CD117-ADC treated animals also remains functionally intact, and animals are able to mount immune responses post LCMV infection and candida challenge. These agents also preserve immune memory and post-treatment, animals were found to maintain immune reactivity to prior LCMV infections. Moreover, these agents appear to be safe apart from mild, transient hepatotoxicity. Given the large clinical need for such conditioning agents, we similarly generated anti-human CD117-ADCs and found that these also inhibited human HSCs in vitro and robustly depleted >99.7% of human HSCs in vivo in humanized mice. We foresee rapid clinical development of such agents and translation of this work to patients. In conjunction with HSC-gene therapy/gene-editing this could result in safe and effective treatment of a wide range of blood and immune diseases ranging from sickle cell anemia to hemophilia to HIV.

45. Liver-Directed Gene Therapy for Hemophilia B with Immune Stealth Lentiviral Vectors

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Lentiviral vectors (LV) are emerging as versatile vehicles of relatively large capacity for stable transgene integration in the genome of target cells. Over the past years, we have developed LV that achieve stable transgene expression in the liver and provide correction of hemophilia, following systemic administration in mouse and dog models of the disease. These LV are designed to stringently target transgene expression to hepatocytes through transcriptional and microRNA-mediated regulation. We have recently generated LV lacking class-I major histocompatibility complexes (MHC-free LV), by genetic inactivation of beta-2 microglobulin in LV producer cells. These MHC-free LV maintain infectivity but significantly reduce the risk of triggering allogeneic immune responses in the recipient (Milani et al., EMBO Mol Med 2017). We then generated LV with increased levels of CD47, a phagocytosis inhibitor, on the vector surface (CD47high LV). These LV show substantially decreased uptake by macrophages and reduced innate immunity activation, following intravenous administration to NOD mice, which can cross-recognize the human CD47. Taking advantage of fluorescent virions and ImageStream, a combined flow cytometry and imaging system, we have set up an *in vitro* LV phagocytosis assay with primary human macrophages and are currently investigating the impact of CD47 and MHC surface content on LV opsonization and phagocytosis, with or without human serum containing LV-binding antibodies and with or without complement inhibitors. In order to evaluate the role of CD47 in LV biodistribution *in vivo* in large animals, we have administered MHC-free or MHC-free/CD47high LV to 6 non-human primates (NHP, 3 for each LV version). We chose *Macaca nemestrina* as recipient, because of the lower restriction to HIV infection as compared to other NHP species. Administration of 7.5e9 T.U./kg LV via peripheral vein was well tolerated, without significant elevation of serum aminotransferases or body temperature and only caused a transient self-limiting leukopenia. Remarkably, human-specific FIX activity reached up to 300% of normal and was nearly 3-fold higher in the CD47high-LV treated animals, showing a much more favorable LV dose-response than observed in mice and dogs. Upon necropsy, we measured vector copies in liver, spleen and major organs of treated animals and found between 0.5 and 1.5 LV copies in the liver accounting for 80-90% of all the retrieved LV copies, showing selective targeting and efficient gene transfer to the liver by LV in NHP. Additional doses of LV are currently being evaluated in NHPs to yield important information on tolerability and human FIX output following LV administration. Overall, our studies support the efficacy and safety of these immune-stealth LV in NHP and position them to address some of the outstanding challenges in liver-directed gene therapy for hemophilia.

46. In Vivo HSC Transduction in Mobilized Mice with Subsequent In Vivo Selection Results in Efficient Expression of Human Gamma Globin in Peripheral Blood Erythrocytes

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Current protocols for HSC gene therapy, involving the transplantation of *ex vivo* lentivirus vector-transduced HSCs into myeloablated recipients, are complex and not without risk for the patient. We have developed a new approach for *in vivo* gene delivery into HSCs that does not require myeloablation and HSC transplantation. It involves injections of G-CSF/AMD3100 to mobilize HSCs from the bone marrow into the peripheral blood stream and the intravenous injection of an integrating, helper-dependent adenovirus (HDAd5/35++) vector system. HDAD5/35++ vectors target CD46, a receptor that is expressed on primitive HSCs. Transgene integration is achieved (in a random pattern) using a hyperactive Sleeping Beauty transposase (SB100x). We demonstrated in adequate mouse models, using GFP as a transgene, that primitive HSCs transduced in the periphery home back to the bone marrow where they persist and stably express GFP long-term (Blood 128:2206 (2016). To achieve high-level transgene marking in differentiated peripheral blood cells, we combined our *in vivo* HSC transduction approach with *in vivo* selection of transduced HSCs using the mgtmP140K mutant gene and low dose O^6BG/BCNU treatment. Here were tested the potential of this approach for the therapy of hemoglobinopathies by ectopic expression of fetal (gamma globin). We generated a HDAd5/35 vector containing the human-gamma globin

gene under the control of a 6kb version of the erythroid specific beta-globin LCR (containing HS1 to HS4 and the beta globin promoter) and the mgmtP140K gene under the control of the ubiquitously active PGK promoter. Mobilized immunocompetent hCD46 transgenic mice were injected IV with the integrating vector system and subjected to four rounds of treatment with low-dose O6BG/BCNU followed by immunosuppression to avoid immune responses against the human transgene product. We detected stable human gamma globin expression in 60-80% of erythrocytes in the peripheral blood of in vivo transduced cells mice over a period of 18 weeks. Human gamma globin levels were 15-40% of endogenous mouse beta globin levels in erythrocytes measured by HPLC and qRT-PCR. The percentage of gamma-globin positive red blood cells was maintained in secondary transplant recipients. The in vivo HSC transduction/selection procedure was safe. It did not alter bone marrow composition or red blood cell morphology. Genome-wide analysis of transgene integration before and after selection showed similar random integration patterns without the emergence of dominant integration sites. Our new approach avoids the collection of HSCs, their transduction *ex vivo*, and subsequent transplantation into myeloablated recipients. It therefore could greatly simplify HSC gene therapy of common diseases. The efficacy of our approach achieved in this study indicates that it would be curative in thalassemia patients. This is underscored by data in a murine beta-thalassemia model that will be presented at this meeting.

47. Safe and Effective Platelet-Targeted Gene Therapy of Hemophilia A Enabled Using Non-Genotoxic, Antibody-Drug-Conjugate Conditioning

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Hemophilia A (HA), a genetic bleeding disorder resulting from a FVIII deficiency, is a prime model for gene therapy. Our previous studies have demonstrated that platelet-targeted FVIII (2bF8) gene therapy through lentiviral modification of hematopoietic stem cells (HSCs) can restore hemostasis and induce immune tolerance in HA ($F8^{null}$) mice. However, sufficient preconditioning is essential to create niches to enable engineered HSC engraftment. Prior preconditioning has involved total body irradiation (TBI) and/or cytotoxic chemotherapy, which is non-targeted and genotoxic. Given the potential concerns associated with these agents developing a protocol with targeted and non-genotoxic preconditioning is desired to increase acceptance of such HSC-based gene therapy. Recently, several novel antibody (Ab)-based preconditioning methods have been developed for allogeneic bone marrow transplantation (BMT). Initially, an antagonistic CD117 Ab was shown to enable engraftment of donor cells in immunocompromised mice (*Science 2007*), and subsequently anti-CD45 and anti-CD117 Abs linked to the protein synthesis toxin saporin (SAP) were shown to enable engraftment in immunocompetent mice (*Nat. Biotech 2016* and *ASH Abstract 2016*). Such antibody-drug-conjugate (ADC) conditioning regimens could be ideal in HSC-based gene therapy, especially for HA. To explore ADC conditioning for 2bF8 gene therapy, ADCs were prepared by combining appropriate biotinylated Abs (CD45 and CD117) with streptavidin-SAP. Sca-1^+ cells were then isolated from BM of CD45.1/$F8^{null}$ donors, transduced with 2bF8 lentivirus (2bF8LV), and transplanted into CD45.2/$F8^{null}$ recipients conditioned with these ADCs or an optimized sub-lethal dose of 6.6Gy TBI. Flow cytometry analysis at 7 weeks (wks) after BMT showed 16±17% (n=10) donor-derived leukocyte chimerism post ADC-treatment, which was lower than post 6.6Gy TBI (75±9%, n=5). By 20 wks after BMT, the chimerism in the ADC group increased to 39±28 % whereas it remained the same in the 6.6Gy group. Although chimerism is a surrogate for gene therapy efficacy, ultimate success would be sufficient FVIII expression to prevent bleeding complications. While chimerism in the ADC-treatment was only 23% of that obtained post 6.6Gy treatment at 7 wks, the level of plt-F8 expression in the ADC group was 0.5±0.8 mU/10^8 plts, which is not significantly different compared to the 6.6Gy group (1.7±1.4 mU/10^8 plts). Plt-F8 expression in the ADC group further increased to 2.5±4.1 mU/10^8 plts at 20 wks after BMT, but it remained similar in the 6.6Gy group. When the tail bleeding test was used to grade phenotypic correction of the $F8^{null}$ coagulation defect, the remaining hemoglobin levels in the ADC group was 76±26%, which was significantly higher than the $F8^{null}$ group (43±12%) and excitingly comparable to the 6.6Gy and WT groups. Furthermore, sustained therapeutic levels of plt-F8 were obtained in transduced recipients with no anti-F8 inhibitors detected even after challenged with rhFVIII, demonstrating that immune tolerance is induced. Thus, we for the first-time show efficient engraftment of gene-modified HSCs without genotoxic conditioning. Specifically we show CD45/CD117-SAP preconditioning enables long-term engraftment of 2bF8-transduced HSCs resulting in sustained therapeutic levels of plt-F8 expression. As multiple similar clinical-grade Abs are in development and being tested in clinical trials, these studies highlight a potential promising, rapidly translatable strategy for treatment of all genetic blood-based diseases. This safe and effective treatment strategy could be especially meaningful for HA patients who are wary of standard preconditioning.

48. Restoration of PLT Structure and Function in Wiskott-Aldrich Syndrome Patients after Gene Therapy Treatment

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Wiskott-Aldrich syndrome (WAS) is an X-linked primary immunodeficiency characterized by thrombocytopenia, eczema,

high susceptibility to develop autoimmune manifestations and malignancies. Although hemorrhages due to thrombocytopenia are a main cause of death in patients, the pathogenesis of platelet (PLT) defect is not completely clarified. Using a conditional knock-out mouse model lacking WASp only in megakaryocytes (MKs) and PLTs, we found that WASp$^{-/-}$ PLTs are mainly targeted by immune cells in the periphery due to their higher expression of P-selectin and CD40L. WAS patients treated with Gene Therapy (GT), using lentiviral vectors (LV) encoding for human WASp under the control of endogenous 1.6 kb promoter, have shown a reduction in the frequency and severity of bleeding, in line with an increase in PLT count and PLT size normalization. Our present study shows that PLTs isolated from WAS patients before GT had a dysmorphic ultrastructure and smaller size compared to adult Healthy Donors (HDs) and showed a higher expression of activation markers on their surface associated with increased level of sCD62P and CD40L in the plasma, in line with murine data. Conversely, PLTs isolated from patients post-GT had size and ultrastructure comparable with HDs, normal activation markers expression at steady state especially at longer follow-up and reduced level of sCD62P and sCD40L in the plasma compared to pre-treatment values. Additionally, the proteomic profile of PLTs showed that GT is able to restore the expression of many protein pathways (cytoskeleton dynamics, metabolism of lipid and glucose, signaling, protein folding) found to be defective in PLTs isolated from pre-GT patients. Our data show that, although in GT patients there is a mix of uncorrected and corrected PLTs, morphology and protein expression normalize. Tests to assess the aggregation ability of GT corrected PLTs are ongoing.

49. Successful Hematopoietic Stem Cell Mobilization and Apheresis Collection Using Plerixafor Alone for Sickle Cell Disease Gene Therapy

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Novel therapies for sickle cell disease (SCD) based on genetically engineered autologous hematopoietic stem/progenitor cells (HSCs) are critically dependent on a safe and effective strategy for cell procurement. In SCD patients, bone marrow (BM) harvest carries a risk of anesthesia-related morbidity, and G-CSF has caused severe adverse events, so alternative options are needed. We conducted a non-randomized study to assess the safety and feasibility of plerixafor mobilization in SCD patients (IND #131740, NCT02989701), and then used the collected cells for transduction optimization and validation for our open NIH-funded SCD gene therapy trial (NCT03282656) based on fetal hemoglobin (HbF) induction by shRNA knockdown of BCL11A. Six adult (19-38 year old) subjects with SCD receiving routine exchange transfusions for stroke prophylaxis were enrolled. Less than 7 days after last transfusion, with HbS levels below 30%, they received a single dose of plerixafor. Three subjects received a lower than standard dose (180μg/kg) and then three received standard dose (240μg/kg). Four to 6 hours later they underwent apheresis targeting 3-5 blood volumes. No subjects experienced vaso-occlusive pain or other SCD-related events. Patients had a transient 2.1- to 3.1-fold increase in peak white blood cell count (maximum 31.9 x 10^9/L) and a transient 2.1- to 3.5-fold increase in peak absolute neutrophil count (maximum 21.3 x 10^9/L). The average peak peripheral CD34+ cell count after 180μg/kg plerixafor was 44/μL (range 31-65/μL), and after 240μg/kg it was 158/μL (range: 27-290/μL); statistical analysis confirmed a plerixafor dose effect. Despite adequate mobilization, the 180μg/kg plerixafor group achieved lower than expected apheresis yields (0.07-1.2 x 10^6 CD34+ cells/kg). After adopting an optimized collection strategy including a higher interface hematocrit and a consistent blood flow using a more stable venous access, yields in the 240μg/kg group were higher with 24.5, 2.9, and 16.4 x 10^6 CD34+ cells/kg collected in each single apheresis session. Immunophenotyping of the collected cells showed enrichment in lineage-negative stem and progenitor cells as compared to pre- and post-plerixafor BM, and enrichment in long-term HSCs and early progenitors as compared to healthy donor G-CSF mobilized cells. Collected cells were then CD34 selected and transduced in large scale with a protocol that was previously validated on G-CSF-mobilized cells from healthy donors and BM HSCs from SCD patients, using a clinical GMP-manufactured lentiviral vector. Importantly, the plerixafor-mobilized SCD cells were efficiently transduced with 3.7-7.5 vector copies per cell and achieved robust HbF induction in their liquid culture progeny. Overall, using the 240μg/kg dose, an optimized collection strategy, and the possibility of repeat collections, plerixafor mobilization appears to be a safe and effective strategy for procuring sufficient numbers of autologous HSCs from transfused SCD patients for use in gene therapy trials.

50. BTK Lentiviral Therapy of XLA Patient Stem Cells Restores B Cell Development and Function in Humanized Mice

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X-linked agammaglobulinemia (XLA) is a rare primary immunodeficiency associated with mutations of the gene encoding Bruton's tyrosine kinase (BTK). The loss of functional BTK leads to near absence of peripheral B cells, serum antibodies and results in recurrent life-threating bacterial and viral infections. Immunoglobulin replacement therapy improves survival but is expensive, non-curative and incompletely protective. Lentiviral (LV) gene therapy using hematopoietic stem cells (HSCs) has the potential to be a curative treatment. We previously developed a SIN- LV construct that used

the human *BTK* promoter (BTKp) to drive codon-optimized BTK cDNA expressionand included an upstream 0.7 kb ubiquitously acting chromatin opening element (UCOE: from the *HNRPA2B1/CBX3* intergenic region) to maintain transgene expression over time (LV 0.7UCOE.*BTK*p.hBTK). In a murine XLA model, LV- transduced murine HSCs restored lineage appropriate BTK expression in B and myeloid cells, reconstituted BM and splenic B cell development, BCR-dependent B cell proliferation and antibody responses. In this current study, we tested the efficacy of this candidate clinical LV to restore BTK function in XLA patient HSCs, using a humanized mouse model. To establish the model, mobilized peripheral blood stem cells (PBSCs) were collected from a series of XLA patients and healthy control subjects using an IRB-approved clinical protocol and equal numbers of XLA or control CD34+ HSC were transplanted into NOD-*scid*-IL2RG^{NULL} (NSG) mice (4 experiments using 4 unique XLA donors). At 12-16 weeks post-transplant, XLA and control recipients exhibited equivalent levels of hCD45^{+} cell engraftment within the BM. In contrast, XLA recipient mice exhibited a differentiation arrest at the pre-B cell stage in the BM and markedly reduced total B cell numbers in the spleen. Splenic B cells exhibited an immature phenotype with the majority lacking sIgM and expressing surrogate light chain. In comparison, PBSCs from control donors differentiated into large numbers of splenic B cells with a transitional or mature phenotype (CD38$^{+/-}$ CD24$^{low/+}$ CD10$^{hi/low}$IgM^{+} IgD$^{-/+}$). Together, these data demonstrate that our NSG model closely mirrors the developmental arrest in subjects with XLA. Next, we evaluated the ability of 0.7UCOE.*BTK*p.hBTK LV gene therapy using XLA HSC to rescue B cell hematopoiesis in this model. XLA PBSCs were transduced with a single hit of BTK LV (MOI of 5-10) following 48 hr pre-stimulation in cytokine-supplemented media (100 ng/ml of hSCF, hTPO and hFLT3) and transplanted into NSG recipients 2 ×10^{6}cells/recipient. At 16 wk post-transplant, recipient animals were sacrificed and analysis of BM and spleen revealed partial to complete restoration of human B cell development (with average viral copy number of 0.4 in BM and 0.5 in spleen). Functionally, splenic B cells from mice receiving LV treated XLA PBSCs exhibited improved Ca^{+2} flux in response to BCR signals, and were able to class switch and develop into plasma cells in vitro. Our findings illustrate the utility of the NSG xenograft model for the preclinical study of XLA gene therapy and support our plans for use of LV 0.7UCOE.*BTK*p.hBTK in a first-in-patient clinical trial for XLA.

AAV Biology

51. RNF121 is a Key Transcriptional Regulator of AAV Genome Expression

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A recent high-throughput screen for essential AAV host factors (Pillay et al., Nature, 2016) identified RNF121, a poorly characterized E3 ubiquitin ligase as a top hit in addition to AAVR. We investigated the role of RNF121 in the AAV life cycle by introducing mutations in the RNF121 gene using CRISPR/Cas9 in a panel of cell lines. Disruption of RNF121 reduced AAV transduction by over two orders of magnitude independent of cell type, AAV serotype or multiplicity of infection. Binding, cellular uptake, and subcellular trafficking assays indicate that AAV particles have no defect in trafficking to the nucleus in the context of the RNF121 knockout (RNF121KO) phenotype. Addition of proteasome inhibitors, transfection of Adenoviral (Ad) helper genes and co-infection with Ad failed to rescue the defective phenotype. Furthermore, transduction with recombinant Adenovirus was not altered by RNF121KO, suggesting this effect is specific to AAV. Transfection of the AAV genome with or without the AAV capsid added in *trans* was not affected, suggesting that association of the AAV genome with the capsid is integral to this phenotype. Transduction with self-complementary (sc) AAV vectors could not rescue the defective phenotype, suggesting second-strand synthesis is not impacted. Strikingly, RNF121KO causes a robust decrease in vector genome mRNA for both recombinant AAV vectors and wild-type AAV implying a defect in AAV genome transcription. These results were further corroborated through RNApol-ChIP. A number of host factors that might be regulated by RNF121 and directly affect AAV genome transcription have been identified by affinity purification mass spectrometry (AP-MS) to further investigate the mechanisms underlying AAV-RNF121 interactions. Our observations characterize a host factor critical for AAV genome transcription and corroborate the evolving notion that the AAV capsid plays a significant role in genome transcription.

52. Mapping and Engineering Functional Domains of the Assembly Activating Protein of Adeno-Associated Viruses

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Adeno-associated viruses (AAV) encode a unique assembly activating protein (AAP) within their genome that is essential for capsid assembly. Studies to date have focused on establishing the role (or lack thereof) of AAP as a chaperone that mediates stability, nucleolar transport, and assembly of AAV capsid proteins. Here, we map structure-function correlates of AAP based on secondary structure and bioinformatics, followed by deletion and substitutional analysis of specific domains, namely, the hydrophobic N-terminal domain (HR), conserved core (CC), proline-rich region (PRR), threonine/serine rich region (T/S) and basic region (BR). First, we establish that the hydrophobic region (HR) and the conserved core (CC) in the AAP N-terminus are the sole determinants for viral protein (VP) recognition. However, VP recognition alone is not sufficient for capsid assembly or conferring serotype specificity. Enhancing the hydrophobicity and alpha-helical nature of the N-terminal AAP region through amino acid substitutions enabled assembly of previously unrecognized VPs into capsids. Interestingly, the adjacent PRR and T/S regions are flexible linker domains that can either be deleted completely or replaced by heterologous functional domains that enable ancillary functions such as fluorescent imaging and precise control over oligomerization. We also demonstrate that the C-terminal BR domains can be substituted with heterologous nuclear and nucleolar localization sequences that

display varying efficiency or with IgG Fc domains for VP complexation and structural analysis. The newly engineered AAPs (eAAP) are more stable and require only about 20% of the original AAP sequence for efficiently supporting AAV capsid assembly. Our study sheds light on the structure-function correlates of AAP and provides multiple examples of engineered AAP that might prove useful for understanding and controlling AAV capsid assembly.

53. A Novel Class of Clade C AAV Capsids Isolated from the Human Population Exhibit Greater Than Ten-Fold Higher Packaging Efficiencies Than AAV2

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Recombinant adeno-associated viruses (rAAVs) are promising vectors for human gene therapy due to their excellent safety profiles and gene delivery efficacies. To date, AAV serotype-2 is the most commonly used and best characterized serotype in clinical studies. Recent FDA approval for the AAV2-based gene-replacement therapy drug, LUXTURNA, represents a milestone for other promising AAV2 platforms. Although, AAV2 is popular for localized gene delivery to eyes and regions of interest in the CNS, it is a "poor producer". Clinical success for AAV2 may therefore be hampered by vector production limitations. Capsid engineering to enhance rAAV effectiveness has mainly focused on manipulation of the 3-fold symmetry to improve tropism, immune evasion, and efficacy. Few studies have examined how other structural elements of the capsid may improve packaging. We therefore aimed to screen our recently identified AAV capsids for variants that exhibit high packaging efficiencies. Analysis of these variants, which were discovered by single molecule, real-time (SMRT) sequencing of >800 human surgical specimens, revealed that they were closely related to AAV2 (Clade B), AAV2/3 hybrids (Clade C), and AAV8 (Clade E) serotypes. By performing crude lysate PCR to screen for capsids that generate high vector titers, we found that 26% of Clade B variants and 29% of the Clade C variants (122 total high performing capsids) exhibit greater packaging efficiencies than the prototypical AAV2 capsid. The high degree of functional capsids discovered demonstrates the practicality of mining for natural capsid variants. Importantly, we have identified a class of Clade C variants with remarkably high vector yields. One such variant exhibits nearly 20-fold greater packaging efficiency than AAV2. Ongoing structural analysis of these and other high-performing AAV-variant groups has enabled identification of capsid sequence features that impact packaging efficiency and serves to guide the further expansion of the AAV tool-kit. Current GMP production schemes for clinical grade rAAV2s are accompanied by significant cost challenges. By exploring the natural diversity of AAV in human tissues, we have discovered a pool of AAV2 and 2/3 variants that exhibit packaging efficiencies that are 10-fold greater than AAV2. Our ongoing efforts not only has the potential to uncover novel AAVs with improved tropism profiles, but hold great promise for discovering new AAV variants that can potentially reduce the cost of vector production - making future AAV-based drugs more affordable. MX, LL, and PWLT are co-contributing authors. YW and GG are co-corresponding authors

54. Interactions of AAV-2 with Its Cellular Receptor, AAVR, Visualized by *Cryo*-Electron Microscopy

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Recently, a receptor essential for cellular transduction by most AAV serotypes has been identified as AAVR, through an unbiased genome-wide screen. AAVR is a multi-domain transmembrane protein that is found predominantly in the *peri*-nuclear region of the *trans*-Golgi network, but transiently at the cell surface, i.e. with trafficking that is similar to AAV's transport through endocytosis towards the nucleus. *Ecto*-domains include five polycystic kidney disease (PKD) domains in series, a domain type that is often involved in protein-protein interactions. Various *ecto*-domain constructs have been expressed heterologously. Several competitively inhibit cellular transduction by AAV and/or have nano-molar avidities when binding between AAVR and AAV is assayed by surface plasmon resonance (SPR). Progress on visualization came first with *cryo*-electron tomography, an electron microscopy (EM) technique in which each virus particle is imaged (in projection) from many directions, allowing 3D reconstruction before signal/noise averaging of particles in the same configuration. This moderate-resolution approach showed receptors bound at a subset of the sites on the virus that would otherwise be considered symmetry-equivalent. AAVR domains proximal to the virus appeared in uniform configuration, but those that were more distal (and not interacting directly), adopted a variety of configurations falling into a handful of discrete classes and reflecting flexibility between the receptor domains. The low occupancy and high heterogeneity of bound receptor are challenges in more conventional EM by single particle analysis (SPA), in which identity between the 60 symmetry-related parts of each capsid would be assumed. Emerging techniques of sub-volume extraction were used classify the configurations surrounding each of the putative 60 binding sites on each particle. The most populous classes for receptor-bound virus showed density for bound PKD domains that was consistent with the lower resolution images from *cryo*-electron tomography. The new reconstructions are at somewhat better than nanometer resolution. Transduction assays with a panel of domain deletion mutants indicate that it is PKD domain 2 that interacts most strongly with AAV-2. Thus, the density seen likely corresponds to PKD2, allowing us to determine which parts of the viral surface interact with the AAVR receptor.

55. Capsid-Glycan Receptor Interactions Influence AAV Transport across the Blood-Brain Barrier

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Our lab recently mapped a neurotropic footprint on AAVrh.10 that is critical for crossing the blood-brain barrier (BBB). We demonstrated that grafting this footprint onto the AAV1 capsid (AAV1RX) enables viral transport across the BBB. Here, we establish through structural modeling that this footprint overlaps in part with the sialic acid (SIA) receptor footprint on AAV1 and is in close spatial proximity to the sulfated lactosamine (LacNAc) receptor footprint of AAVrh.10. We generated a panel of AAV capsid variants with altered SIA footprints that were characterized through comprehensive *in vitro* binding, transduction, neuraminidase sensitivity and lectin inhibition assays across a panel of CNS-derived cell lines (of neuronal, glial and brain microvascular endothelial origins). These studies revealed three functional subgroups based on high, moderate and low sensitivity to neuraminidase/lectin treatment. Systemic administration of this panel of AAV variants in mice, followed by immunohistochemical analysis of GFP transgene expression in the brain, corroborated distinct abilities of the three subgroups to traverse the BBB. Specifically, capsids with ablated SIA-binding can cross the BBB, although only transduce the CNS with low-to-moderate efficiency. At the other end of the spectrum, AAV capsids with strong binding affinity to SIA do not cross the BBB after systemic administration; on the contrary, they show high levels of vascular transduction and are sequestered within the liver. The AAV1RX variant, which shows an intermediate SIA-binding phenotype, is not only effective at crossing the BBB, but also transduces CNS tissue at levels only slightly lower than AAVrh.10. These latter results might suggest potential involvement of other host factors such as interactions with LacNAc, the AAVrh.10 receptor. We also observed lower liver transduction levels for capsids with reduced SIA-binding affinity. Additionally, our data indicates that SIA-binding affinity is a key determinant for transducing and sequestration within the vasculature. We postulate a "Goldilocks" model, wherein the ideal glycan binding affinity (in conjunction with other contributing factors) can profoundly impact the ability of AAV capsids to traverse the BBB and transduce the CNS. Such a scenario is further corroborated by earlier reports with autonomous parvoviruses and polyomaviruses, which also show increased neurovirulence and spread attributable to reduced SIA binding affinity. Taken together, our studies shed light on the mechanisms governing AAV CNS tropism and provide a structure-guided platform for fine-tuning AAV vector transport across the BBB for gene therapy of neurological disorders.

56. Systematic Functional Characterization of the AAV Capsid Fitness Landscape

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Engineered AAV capsids can make current therapies more effective while enabling the treatment of many new diseases. To better understand capsid sequence-function relationships and facilitate capsid engineering, we systematically created a barcoded library of all possible single codon substitutions, insertions and deletions across the entire AAV2 capsid (94,080 mutations in total). This library contains all possible first-order changes to the protein, enabling us to create high-resolution data-rich maps of the AAV capsid fitness landscape for functions such as capsid assembly, thermostability, heparin binding, infection *in vitro* and biodistribution *in vivo*. Our results reveal surprising new functions of the VP1 region, provide the basis for a mechanistic understanding of sequence-function relationships, and reveal empirical design rules for engineering tissue tropism. We expect that the experimental characterization of fitness landscapes will become an essential tool for understanding AAV biology and for capsid engineering.

57. A Proximity-Based Proteomics Approach to Identify Cellular Proteins Interacting with AAV Assembly Activating Protein (AAP)

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The adeno-associated virus (AAV) assembly-activating protein (AAP) promotes capsid assembly. However, how AAP facilitates capsid assembly still remains poorly understood. In particular, it is not known which host cellular proteins participate and how they are involved in the capsid assembly process. To identify host cellular proteins that interact with AAP2, we conducted co-immunoprecipitation (Co-IP) experiments with AAP2 as a bait followed by LC-MS/MS, only to result in the identification of AAV2 VP protein as the sole meaningful Co-IP'ed protein. We speculated that the reason why the Co-IP approach is challenging in identifying AAP-interacting proteins is due to possibly weak and/or transient nature of protein interactions that might be lost during Co-IP. To overcome this potential problem, we have started exploring proximity labeling-based approaches that provide spatially restricted proteome data. Here we report a successful application of BioID to AAV proximity proteomics and show preliminary data on a set of cellular proteins that potentially interact with AAP2. In our BioID approach, a promiscuous bacterial biotin ligase mutant (BirA*) is fused to the AAP2 protein either at the N- or C-terminus (B-AAP2 or AAP2-B). Such AAP2 and BirA* fusion proteins are expected to biotin-label any proteins residing in close proximity to AAP2 in cells, which can be effectively pulled down by streptavidin beads under stringent conditions such as high salt to reduce non-specific interactions. In this study, we constructed a total of 6 AAP2 and BirA*

fusion proteins (B-AAV2, B-NLS-AAP2, B-NLS/NoLS-AAP2, AAP2-B, AAP2-B-NLS and AAP2-B-NLS-NoLS) and their controls (B, B-NLS and B-NLS-NoLS), where NLS is the Nuclear Localization Signal (from SV40) and NLS-NoLS is the Nucleolar Localization Signal (from AAP2). The AAP2 fusion proteins and their control fusion proteins were validated for sub-cellular localization, protein expression, biotin ligase activity and capsid assembly function. These proteins were expressed in HEK293 cells with or without AAV2 VP3 protein by plasmid transfection and labeled with biotin exogenously added into the culture medium. Total proteins were recovered from the cells 48 h post-transfection and subjected to a pull-down with streptavidin beads followed by LC-MS/MS. This BioID approach identified at least 33 cellular proteins as candidates for AAP2-interacting proteins in two independent experiments. Gene ontology enrichment analysis of these proteins revealed significant enrichment for nucleolar proteins involved in ribosome biogenesis, validating the proximity-based assay. One protein in the candidate list that has garnered our attention is a deubiquitinating enzyme (DUB) enriched in the nucleolus. Preliminary data obtained with a dominant-negative DUB mutant and a proteasome inhibitor has indicated that loss-of-function of the DUB results in retention of ubiquitin tags on AAV VP thereby facilitating VP's proteasomal degradation, and hence lowering the quantity of assembled capsids. These observations highlight a novel role of AAP in regulating stability of VP by spatially restricting VP to interact with a cellular DUB in the site where capsid assembly takes place. Thus, our study demonstrates that BioID is a promising method to study the cellular biology of AAP and capsid assembly.

Translational and Clinical Progress in Neurological Disease

58. AVXS-101 Phase 1 Gene Therapy Clinical Trial in SMA Type 1: Event-Free Survival and Achievement of Developmental Milestones

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Spinal muscular atrophy (SMA) is a devastating, monogenic neurodegenerative disease. Children with its most severe form, SMA Type 1 (SMA1), will never sit unassisted or maintain head control. A natural history study of SMA1 reported that none achieved an Infant Test of Neuromuscular Disorders (CHOP-INTEND) score of ≥40 by 6 months of age (1 transient exception) and 92% died or required permanent ventilation by 20 months. This trial explores safety and efficacy of a single intravenous administration of gene therapy in SMA1. AVXS-101 delivers the SMN gene in a single intravenous dose via the AAV9 viral vector, which crosses the blood-brain barrier. In this phase 1 trial, 15 patients with SMA1 confirmed by genetic testing (with 2x*SMN2* copies) were enrolled. Patients received an intravenous dose of AVXS-101 at the low dose (Cohort 1, n=3) or proposed therapeutic dose (Cohort 2, n=12). The primary objective was safety and secondary objectives included survival (avoidance of death/permanent ventilation) and ability to sit unassisted (video confirmed by external independent reviewer). CHOP-INTEND scores and other motor milestones are additional objectives. AVXS-101 appeared to have a favorable safety profile and to improve survival (August 7, 2017). All 15 patients were alive and event-free at 20 months of age and did not require permanent mechanical ventilation. Patients in Cohort 2 demonstrated improvements in motor function: 11/12 had achieved CHOP-INTEND scores ≥40 points and a mean increase of 24.6 points from a mean baseline of 28.2 points, 11/12 were able to sit unassisted for at least 5 seconds, 10 for at least 10 seconds, and 9 for at least 30 seconds; 11/12 achieved head control and 9 could roll over. Two patients were able to crawl, pull to stand, stand independently, and walk independently. Asymptomatic elevated serum aminotransferase levels occurred in 4 patients and were attenuated by prednisolone. In contrast with the natural history, a one-time intravenous administration of AVXS-101 appeared to demonstrate a positive impact on the survival of both cohorts and a dramatic, sustained impact on motor function of Cohort 2: 11/12 patients achieved CHOP-INTEND scores and motor milestones rarely or never seen in this population. No waning of effect or clinical regression in motor function was reported up to the August 7, 2017 data cut. A clinical update of the 24-month safety follow-up will be given at the time of presentation.

59. Dose Escalation Gene Therapy Trial in Children with AADC Deficiency

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Aromatic L-amino acid decarboxylase (AADC) deficiency is a rare autosomal recessive genetic disorder that causes deficient synthesis of the monoamine neurotransmitters dopamine and serotonin. The condition typically presents in infancy with hypokinesia, hypotonia, oculogyric crises (OGC), dystonia, autonomic symptoms, sleep disruption, and motor developmental delay. Affected children with AADC deficiency experience chronic, severe physical and intellectual disability, and most patients derive little or no benefit from currently available medical therapies. The objective of this clinical trial is to determine the safety and efficacy of 2 doses of adeno-associated virus serotype 2 (AAV2)-hAADC delivered to the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA). To our knowledge, this is the first prospective study that utilizes axonal transport to deliver a transgene to projection regions (striatum and nucleus accumbens), which control the motor and mesolimbic systems. Our

MR-guided AAV delivery platform permits AAV delivery to the target with submillimeter accuracy and for monitoring of vector delivery in real-time. Three children (2 female and 1 male; ages 9, 8, and 5 years, respectively) were treated with a low dose of vector (1.3×10^{11} vector genomes) and have been followed for 3 to 12 months after surgery. Data consists of clinical examinations, ^{18}FDOPA-PET/MRI scans and CSF analysis of biomarkers. Real-time MR data confirmed accurate targeting and close to 70-100% coverage of the SNc/VTA. There have been no adverse events related to the surgical intervention. All children developed mild to moderate involuntary movements (dyskinesia) 1 month after surgery. Movements peaked between 1 and 3 months and then improved. Gene transfer resulted in complete cessation of OGC in 2/3 patients, and a significant reduction in the severity and duration of episodes in the third patient. Sleep and mood markedly improved in all 3 subjects. Motor function as measured by the GMFM-88 scale improved by 3-5 points in the 3 subjects at 3-6 months. One subject attained the ability to sit independently and take some steps with support at 8 months. CSF homovanillic acid (HVA) increased in all subjects from less than 10% at baseline to 27-32% of the lower limit of normal 3-6 months post-gene transfer. ^{18}FDOPA PET demonstrated increased uptake in the midbrain, caudate and putamen. Our findings demonstrate that AADC gene transfer to SNc/VTA is safe, and produces an improvement in dystonic symptoms, mood, and motor function in children with AADC deficiency. We hypothesize that selecting nuclei in the midbrain that normally innervate the striatum may result in more physiologic dopaminergic transmission than targeting (non-dopaminergic) medium spiny neurons in the striatum for gene delivery. Safety data to date support the plan to increase the dose in the next cohort of children.

60. Gene Therapy Improves Cerebral White Matter Microstructures in Patients with Aromatic L-Amino Acid Decarboxylase Deficiency

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Background: Children with aromatic l-amino acid decarboxylase (AADC) deficiency suffer from severe motor dysfunction because of dopamine deficiency in the putamen. Infusion of an adeno-associated viral vector to the putamen restored dopamine levels, and patients exhibited dramatic improvements in motor function. In this study, we examined brain white matter microstructures in these patients. **Method:** Brain magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) studies were performed in patients before and 12-months after gene therapy. White matter tractography was performed for 76 tracts using tract-based automatic analysis. **Results:** Eight patients (1.67 to 8.42 years of age) were enrolled in this study, and all of them improved in motor function after gene therapy. Before gene therapy, MRI studies reveled mild dilatation of lateral ventricles and DTI studies revealed low total (76 tracts) mean tract fractional anisotropy (FA) values. After gene therapy, there was no significant change in MRI, but total mean FA values increased (p=0.012) and the increment was inversely related to age (C.C.=−0.738; p=0.036). Tract-wise analysis demonstrated more improvements in right- than left-side tracts, and the right-side tracts improved included most of the association tracts, corticospinal tracts to the mouth, throat, hand, and toe, thalamic radiation to the motor and premotor cortices, and corpus callosum connecting the motor and premotor cortices. **Conclusion**: An increase in motor activity brought by gene therapy triggers improvements in the microstructures of white matter tracts involving the motor and premotor cortices. Neuroplasty is probably required because young patients improved better. The preferential improvements of the right side tracts further implies brain motor function laterality.

61. First Gene Supplementation Therapy for *CNGA3*-Linked Achromatospia

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Achromatopsia type 2 (ACHM2) is a genetically and clinically well-defined inherited retinal disorder caused by mutations in the *CNGA3* gene. ACHM2 patients present with severely impaired daylight vision, poor visual acuity, photophobia, nystagmus, and lack the ability to discriminate colors. We developed a recombinant adeno-associated virus (AAV) vector for gene supplementation therapy of ACHM2. The vector expresses full length human *CNGA3* under control of the human cone arrestin promoter and was packaged with AAV8 capsid. A first in man interventional phase I/II clinical trial (NCT02610582) was conducted focusing on safety and efficacy of a single subretinal injection of rAAV8.CNGA3 in patients with ACHM2. The study was designed as open label, monocentric, exploratory, dose-escalation trial. A total number of nine patients were enrolled and three patients were treated per dose group ($1x10^{10}$, $5x10^{10}$, and $1x10^{11}$ total vector genomes (vg) of AAV8.hCNGA3). Vector administration was achieved by a single subretinal injection targeting the central retina and involved temporal detachment of the foveomacular region. Safety as primary endpoint was assessed by clinical examination and best corrected visual acuity (BCVA), vital signs, blood chemistry (CRP, ESR, blood counts), and immunopathology assays over a period of 12 months. Biodistribution and shedding was monitored in blood, urine, saliva and lacrimal fluid. The primary endpoint was met with an excellent safety profile and no serious adverse event. Ocular adverse events were either unrelated or associated with the surgical procedure, but not the study drug. Analysis of vital signs, blood chemistry, immunopathology and shedding supported the excellent safety profile. Although the study was not designed and powered to demonstrate efficacy, exploration of secondary endpoints support the notion that the treatment could improve clinical features of ACHM. Despite foveomacular detachment a slight, but significant increase in best corrected visual acuity (BCVA) was measured (-0.05±0.02 logMAR [mean±se; p = 0.038]).

In conclusion, the first clinical gene therapy trial for achromatopsia in man was well tolerated, safe and provides evidence that ACHM2 can be treated by gene supplementation.

62. Immune Blockade in CNS Gene Therapy Improves Safety and Clinical Outcome

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A recombinant AAV vector has been engineered to carry the human aspartoacylase (ASPA) gene expressed from a modified CMV-enhancer chicken β-actin (CB6) promoter. The construct has been shown to produce ASPA in animal models of Canavan disease, which closely match the human study. The clinical study is an open label, expanded access trial administering the rAAV9-CB6-ASPA gene by simultaneous systemic and intracerebroventricular routes to a single Canavan patient at 18 months of age. The subject received immune modulation to transiently ablate B-cells (Rituximab) and modulate T-cell response (Sirolimus) prior to the initial exposure to AAV9. Given the severe AspA mutations of the subject and baseline AAV Ab-titer showing no prior exposure to AAV, the regimen employed would allow for subsequent exposure to the therapeutic vector if needed and block any immuno-toxicity in the CNS at the time of initial delivery. The goal of this study was to measure the safety and efficacy of AAV-mediated gene therapy as a treatment approach for neuronal pathology in Canavan disease. Results at 9 months following dosing shows safety and tolerability of the gene replacement treatment as measured by serum chemistries and hematology, urinalysis, physical assessments, whole blood assay for vector genomes, immunologic response to AAV, as well as reported subject symptom history. Further, positive change from baseline for levels of brain NAA, brain water content, fractional anisotropy and morphology, clinical status and functional motor/cognitive outcomes were also observed. Results of this study demonstrate that gene therapy can be a successful approach for the treatment of a complex neurodegenerative condition such as Canavan disease. Furthermore, for the first time, simultaneous multiple routes of vector delivery were successfully implemented to reach the target dose. The immune management used in this study would allow for subsequent exposure of the study agent. This approach is relevant to other genetic diseases to reach both peripheral target organs as well as the brain.

63. *AADC* Gene Therapy for Advanced Parkinson's Disease: Interim Results of a Phase 1b Trial

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Objective: Evaluate the safety of AAV gene therapy with human aromatic L-amino acid decarboxylase (AADC) using intraoperative MRI to improve delivery. Background: Levodopa therapy for Parkinson's disease (PD) becomes less effective over time, possibly due to progressive loss of AADC, which converts levodopa into dopamine. Methods: Fifteen subjects with advanced PD received bilateral transfrontal infusions of AAV-hAADC vector admixed with gadoteridol to facilitate the intra-operative visualization of coverage of the putamen. Subjects in Cohort 1 received up to 450 µl/putamen at a concentration of 8.3×10^{11} vg/ml, Cohort 2 received up to 900 µl/putamen at 8.3×10^{11} vg/ml, and Cohort 3 received up to 900 µl/putamen at 2.6×10^{12} vg/ml. ^{18}F-dopa PET was obtained at baseline and 6 months to assess gene expression. Results: Treatment was well tolerated with no vector related SAEs. There was one post-procedure pulmonary embolus which resolved. In cohorts 1, 2 and 3 respectively: (a) coverage of the putamen by vector was 21%, 34% and 42%; (b) ^{18}F-dopa PET signal increased by 13%, 56% and 79%; (c) daily levodopa equivalent doses decreased by 14%, 34% and 42% at 6 months (and remained generally stable thereafter), and (d) On-time without troublesome dyskinesia, as reported in motor diaries, increased by 1.6 and 3.3 hours in cohorts 1 and 2 at 12 months, and by 1.5 hours at 6 months in cohort 3. UPDRS motor and PDQ-39 scores showed similar dose-dependent trends. Conclusion: These interim results show that AAV-hAADC gene therapy using intraoperative MRI is well-tolerated, provides dose-dependent gene expression and potential clinical efficacy.

64. First-In-Human Intrathecal Gene Transfer Study for Giant Axonal Neuropathy: Interim Analysis of Efficacy and Review of Safety

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GAN is a rare pediatric neurodegenerative disorder affecting the central and peripheral nervous system. Recessive GAN mutations cause dysfunction of gigaxonin, a cytoskeletal regulatory protein, leading to progressive sensorimotor and optic neuropathy, CNS involvement, and respiratory failure with death by the 2nd to 3rd decade of life. We report on a single site, phase I, non-randomized, open label dose escalation gene transfer study for GAN (NCT02362438), which also represents the first-in-human intrathecal (IT) AAV9 mediated gene transfer trial for any indication, with the longest follow-up to date. Nine GAN patients have been dosed at three dose levels (ranging from 3.5x10^13 vg to 1.8x10^14vg) with scAAV9-JeT-GAN, with follow up data out to 24 months post gene transfer. We present here a review of safety data in the study, as well as interim study analysis data evaluating efficacy in this study. We utilize natural history study data for GAN for comparison of outcome measures in this analysis. Outcome measures of interest include: motor function measure 32 (MFM32), neuropathy impairment score (NIS), Friedrich's Ataxia Rating Scale (FARS), myometry, grip and pinch strength, timed testing (where applicable, electrophysiologic (nerve conduction) data, and neuroimaging data. We review this data to highlight the overall safety and feasibility of an IT route of AAV9 based gene transfer. We further review efficacy in this study to highlight several key aspects: 1) feasibility for adequately targeting the nervous system, 2) preferred IT dosing regimens needed for effective transduction, 3) relevance of baseline neurologic impairment and disease progression in patient selection and stratification. We also discuss here the utilization of immunosuppressive regimens in this study and their impact on safety in this trial. This study represents a proof of concept for the employment of IT gene transfer as a strategy for gene replacement in disorders affecting the central nervous system.

Cell Therapies

65. hESC-Derived Striatal Cells Generated in a 3D Hydrogel Promote Recovery in a Huntington's Disease Mouse Model

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Huntington's disease (HD) is an inherited neurological disorder characterized by the progressive degeneration of striatal medium spiny neurons (MSNs), which eventually leads to fatal deficits in movement, cognition, and behavior. One promising approach for treating HD is cell replacement therapy, where lost cells are replaced by striatal cells or progenitors derived from human pluripotent stem cells (hPSCs), in order to re-establish the functions lost in disease. While remarkable prior work paved the way to generate striatal cells from hPSCs for application towards HD treatment, as with many stem cell differentiation processes the current state-of-the-art methods for striatal cell production rely on 2D culture systems that typically include poorly defined components, limit scalability, and have yielded mixed therapeutic outcomes in animal models of HD. To take a next step towards clinical translation, here we develop an approach for the efficient generation of striatal progenitors from human embryonic stem cells (hESCs) within a fully defined and scalable PNIPAAm-PEG 3D hydrogel. Specifically, a 2-fold higher fraction of DARPP32$^+$ striatal neurons were generated in the 3D biomaterial, which demonstrated functional electrophysiological maturity 30 days earlier than cells differentiated on conventional 2D surfaces. Following their rapid derivation, transplantation of 3D-derived striatal progenitors into a transgenic mouse model of HD slowed disease progression, improved motor coordination, and significantly increased lifespan by 19%. Importantly, transplanted cells developed an MSN-like phenotype and formed synaptic connections with host cells. Our results illustrate the potential of scalable 3D biomaterials for generating striatal progenitors for HD cell therapy.

66. CRISPR-Mediated Genetic Engineering of Human Mesenchymal Stromal Cells for Therapeutic Protein Delivery in Chronic Wounds

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Human mesenchymal stromal cells (hMSCs) have been used for a wide variety of clinical indications. When infused systemically or injected locally in injured tissues, MSCs survive transiently and secrete pro-regenerative factors in a "hit-and-run" fashion, although the specific nature of the regenerative properties is not well understood and the overall clinical results have not been great. MSCs as cellular cargos for therapeutic proteins offer *in vivo* protection from proteolytic environments. Using genetic engineering, it might be possible to generate more therapeutically potent to MSCs for regenerative medicine applications. We utilized a gene integration platform composing of MS-modified guide RNAs, Cas9 mRNAs, and rAAV6 vectors to engineer selectable, therapeutic protein-secreting hMSCs as treatments for chronic wounds. To improve cellular regeneration, we designed bi-cistronic *PDGFB-t2A-GFP* and *VEGFA-t2A-GFP* overexpression cassettes to integrate at the *HBB* locus, an intended safe harbor in hMSCs. We obtained 10.5% and 11.0% integration of *PDGFB* and *VEGFA* expression cassettes, respectively, following by a FACS enrichment of GFP+ cells to > 85% purity. Conditioned media of enriched hMSCs contained more than 10-fold and 29-fold elevated PDGF-BB and VEGFA, respectively, as compared to those of wildtype cells. In a well-established *db/db* mouse diabetic wound healing model, we injected enriched PDGF-BB and VEGFA-secreting cells into the sub-dermal layer of 6-mm full-thickness punch biopsy wounds (n = 10), and quantified the kinetics of healing over 4 weeks, until complete closure. For PDGF-BB- and VEGFA-secreting hMSC-treated groups, midpoints of healing were shortened by 24.4% ($p < 0.05$) and 28.0% ($p < 0.01$), respectively, when compared to vehicle controls. Complete closure was accelerated by 14.3% ($p < 0.001$) and 22.8% ($p < 0.01$),

respectively. Skin sections of closed wounds revealed increased granulation tissue area by 84.6% ($p < 0.05$) in wounds treated with PDGF-BB-secreting hMSCs, thus demonstrated both the effectiveness of our genome editing approach in hMSCs and the functionality of engineered hMSCs as *in situ* synthesis machinery. To investigate survival of engineered hMSCs in wounds, we injected engineered firefly luciferase (F-luc)-expressing cells into wound beds (n = 6) and tracked bioluminescence kinetics. We detected F-luc activities for up to 9 days, locally at the injection sites. More importantly, there was a clear lack of immunogenicity following a second delivery, highlighting the feasibility for re-administrations. We further explored the ability to embed our VEGFA-secreting cells in a hydrogel matrix (n = 10), and found no significant difference in healing kinetics from direct injection ($p > 0.99$). In summary, we have established an effective proof-of-concept sgRNA/Cas9 mRNA/rAAV6 genome editing platform towards engineering primary hMSCs as cell therapy products. We have successfully engineered protein-secreting hMSCs by precisely integrating protein overexpression cassettes into the *HBB* safe harbor. Injected engineered hMSCs briefly persisted locally at injection sites, and functionally exerted intended effects in *db/db* mice. We further showed that engineered hMSCs elicit no immunogenicity, and can be injected directly or applied in embedded hydrogel, highlighting compatibility with different administering methods and routines for a range of therapeutic protein treatments.

67. Delivery of Zinc Finger Artificial Transcription Factors Using Engineered Mesenchymal Stem Cells Results in Re-Activation of UBE3A Throughout the Mouse Brain

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Angelman Syndrome (AS) is a genetically inherited neurodevelopmental disorder characterized by impaired cognitive development, lack of speech, seizures, and motor ataxia. The genetic cause for AS is usually due to a *de novo* deletion of the maternal *UBE3A* gene. Additionally, brain-specific postnatal imprinting of the intact paternal *UBE3A* gene results in complete loss of UBE3A in mature neurons due to the presence of a long antisense transcript driven by the neighboring *SNURF/SNRPN* promoter. Our group has previously shown re-activation of the paternally silent *Ube3a* gene in the brains of a mouse model of AS following i.p. injection of a KRAB-fused Zinc Finger artificial transcription factor (referred to as S1K) targeted to the *Snurf/Snrpn* promoter. This treatment effectively silenced expression of the antisense transcript, allowing UBE3A to be expressed. As an alternative delivery method, we have engineered S1K-secreting bone-derived mesenchymal stem cells (MSC) following lentiviral reprogramming. MSCs have demonstrated a strong safety profile in human clinical trials and exhibit several innate therapeutic effects, such as immune system modulation, homing to injury, and cytokine release into damaged microenvironments. The ability of MSC to transfer larger molecules such as organelles are suggestive of their potential usefulness as delivery vehicles for artificial transcription factors such as zinc fingers. Here we show a series of *in-vitro* and *in-vivo* AS model experiments that demonstrate highly efficient *Ube3a* reactivation by the reprogrammed MSCs. We engineered mouse MSCs to secrete S1K as confirmed by uptake into Neuro2a cells with MSC-S1K conditioned media via fluorescent microscopy. We then bilaterally transplanted MSC-S1K into the hippocampus of 8-week old *Ube3a:YFP* reporter mice. At 3-weeks post-transplantation, we observed significant re-activation of the silenced *Ube3a:YFP* gene compared to controls via IHC and western blotting in the hippocampus, cerebellum, and cortex. Future experiments will evaluate improvement of phenotypic outcome measures in the AS mouse model. Currently, we report the first-of-its-kind use of MSCs as a delivery platform for epigenetic modifiers in neurologic disease - expanding the therapeutic potential of both systems for future genetic diseases.

68. Functionally-Relevant Morphological Profiling Using Visne Reveals Emergent Morphological Subpopulations of IFN-γ-Stimulated MSCs That Predict Immunosuppression

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Introduction: Although efforts have been made to identify quality attributes of multipotent stromal cell (MSC) function, current strategies to characterize MSCs have been unable to effectively address MSC heterogeneity, perhaps leading to inconsistent clinical outcomes. We previously developed techniques to identify population-based morphological features predictive of MSC osteogenic and immunosuppressive capacity and sought to expand upon these methods by quantitatively assessing heterogeneity based on morphological subpopulations (SP_γs). We utilized viSNE (visual stochastic neighbor embedding) to visualize high dimensional morphological data of interferon-gamma (IFN-γ) stimulated MSCs to facilitate identification of SP_γs that predict immunosuppression. Methods: Morphological data from ~$3x10^5$ single cells was uploaded to Cytobank and viSNE was performed. SP_γs were identified for each IFN-γ concentration and both SP_γ total cell number and frequency were determined for each MSC sample and correlated with their immunosuppressive capacity (AUC, where lower AUC indicates greater immunosuppression). Results and Discussion: viSNE permitted visualization of high dimensional morphological data, which allowed for identification of distinct SP_γs using a contour density plot (Fig. 1A,B). Representative cells from different SP_γs illustrate the ability of viSNE to identify distinct phenotypes (Fig. 1C). We observed donor and passage-dependent differences in SP_γ number and found that immunosuppressive capacity was strongly correlated with $SP_\gamma 9$ number as MSC samples with greater immunosuppressive capacity (lower AUC) possessed more $SP_\gamma 9$ cells (Fig. 1D, $p<10^{-5}$). Linear models based on $SP_\gamma 9$ number were then used to predict the immunosuppressive capacity of new MSC samples (Fig. 1E), which also demonstrated the potential to create functional

and morphological standard curves for comparing different MSC samples. Finally, we analyzed the dynamic response of MSCs to IFN-γ by tracking cell morphology with time and showed that exposure of MSCs to IFN-γ reveals emergent morphological phenotypes not apparent in unstimulated MSCs (Fig. 1F). Conclusion: Using viSNE, we could identify morphologically-distinct MSC subpopulations that could be used to predict immunosuppression, as well as serve as a novel analytical platform for assessing functional heterogeneity. Functionally-relevant morphological profiling represents a powerful tool for assessing MSC functional heterogeneity and could be readily adapted to other therapeutic cell-types and applications to improve manufacturing of high quality cellular products.

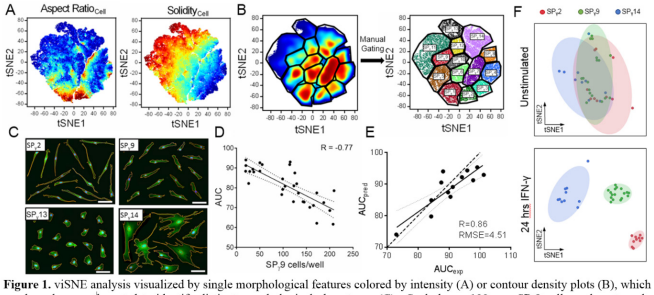

Figure 1. viSNE analysis visualized by single morphological features colored by intensity (A) or contour density plots (B), which can be subsequently gated to identify distinct morphological phenotypes (C). Scale bar = 100 μm. SP_i9 cell number strongly correlates with immunosuppressive capacity (D) and can be used to predict AUC (AUC_{pred}) of new experimental groups (E). (F) Dynamic morphological profiling reveals the emergence of SP_i8 following IFN-γ stimulation.

69. Surface Mobility and Cluster Formation of Various Melanoma Associated Antigens Modulates Car T Cell Activation

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Chimeric antigen receptor (CAR) modified T cells are hailed as a revolutionary breakthrough in the field of oncology. CAR T cells were first applied, with outstanding success, in the treatment of various leukaemias, however solid cancers failed to respond significantly to CAR T cell therapy. Understanding the molecular mechanisms of action of chimeric antigen receptors might help us overcome some of the obstacles. Human high molecular weight-melanoma-associated antigen (HMW-MAA) and melanotransferrin (p97) have similar expression patterns on the tumor cell surface; however, anti-p97 CARs appear to induce stronger specific cytolytic activation of T cells than CARs targeting HMW-MAA with similar affinity and specificity. In this study, we have investigated whether different cluster formation of melanoma-associated antigens on the target cell has any effect on synapse formation, or the cytolytic function on the effector T cell. Using confocal microscopy and fluorescence correlation spectroscopy on immunostained Melur melanoma target cells we found that p97 antigens form smaller clusters (Cluster diameter (CD): ~280 nm) in the membrane and diffuse more rapidly (diffusion correlation time (DCT): 44±22 ms) than HMW-MAA molecules (CD:~740 nm; DCT: 124±42 ms). At the same time, spatial cross-correlation analysis has shown significant overlap (C=0.46±0.06) between HMW-MAA clusters and GM1 positive membrane microdomains (lipid rafts), and less overlap (C=0.21±0.03) for p97. At the same time, p97 specific CAR T cells form larger synapses in cocultures with non-treated Melur cells than HMW-MAA CAR Ts. Following 30 minutes 5mM methyl-β-cyclodextrin (MBCD) treatment of melanoma cells, GM1 domains were disrupted, which resulted in decreased co-localization of HMW-MAA with lipid rafts (C=0.24±0.09) and increased diffusional mobility. MBCD treatment of target cells also improved synapse formation of HMW-MAA specific CAR T cells. In summary, the smaller, more mobile, raft independent p97 antigen clusters induce better CAR T cell activation than the larger, less mobile, raft anchored HMW-MAA. MBCD treatment of melanoma cells removes cholesterol from lipid rafts and disrupts large HMW-MAA clusters, resulting in larger CAR specific immune synapses and more potent T cell activation. Our in vitro findings therefore suggest that a thorough biophysical characterization of the target antigen mobility and association could become an important step in designing CAR T cell therapies.

70. Improving Functional Maturation of Human Pluripotent Stem Cells Derived Cardiomyocytes through Metabolic Understanding

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In vitro differentiation of human pluripotent stem cells into cardiomyocytes (hPSC-CMs) is a crucial process to enable their application in cell therapy and drug discovery. Nevertheless, despite the remarkable efforts over the last decade towards the optimization of cardiac differentiation protocols , generated hPSC-CMs are still immature, closely reminiscent of fetal cardiomyocytes in what regards structure, metabolism and function. In this study, we aim to overcome this hurdle by devising a novel metabolic-based strategy to improve hPSC-CMs generation and functionality. Noteworthy, we integrated structural and functional analyses of hPSC-CM with powerful "omics" technologies (proteomics, transcriptomics, metabolomics and fluxomics) as complementary analytical tools to support process optimization and product characterization. We relied on the aggregation of hPSC-derived cardiac progenitors to establish a scalable differentiation protocol capable of generating highly pure CM aggregate cultures. Whole-transcriptome analysis and ^{13}C-metabolic flux analysis demonstrated that a three-dimensional (3D) and agitated-based culture environment enhances metabolic maturation of hPSC-CMs. When compared to static monolayer, 3D cultures of hPSC-CMs displayed down-regulation of genes involved in glycolysis and lipid biosynthesis and increased expression of genes involved in OXPHOS. Accordingly, 3D hPSC-CMs had lower fluxes through glycolysis and fatty acid synthesis and increased TCA-cycle activity. We then assessed if alteration of culture medium composition to mimic *in vivo* substrate usage during cardiac development improved further hPSC-CM maturation *in vitro*. Our results showed that shifting hPSC-CMs from glucose-containing to galactose- and fatty acid-containing medium promotes their fast maturation into adult-like

CMs with higher oxidative metabolism, transcriptional signatures closer to those of adult ventricular tissue, higher myofibril density and alignment, improved calcium handling, enhanced contractility, and more physiological action potential kinetics. "-Omics" analyses showed that addition of galactose to culture medium improves total oxidative capacity of the cells and ameliorates fatty acid oxidation avoiding the lipotoxicity that results from cell exposure to high fatty acid levels. This study demonstrated that metabolic shifts during differentiation/maturation of hPSC-CM are a cause, rather than a consequence, of the phenotypic and functional alterations observed. The metabolic-based strategy established herein holds technical and economic advantages over the existing protocols due to its scalability, simplicity and ease of application. Improved maturation and functionality of *in vitro* generated hPSC-CM will excel their application in cell therapy, drug discovery and cardiac disease modeling. Funding: This work was supported by FCT-funded projects CardioRegen (HMSP-ICT/0039/2013), CARDIOSTEM (MITPTB/ECE/0013/2013) and Netdimond (SAICTPAC/0047/2015). iNOVA4Health Research Unit (LISBOA-01-0145-FEDER-007344) is also acknowledged.

71. Highly Efficient Chondrogenic Differentiation of Human iPSCs and Purification via a Reporter Allele Generated by CRISPR-Cas9 Genome Editing

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The chondrogenesis of human induced pluripotent stem cells (hiPSCs) enables the engineering of hyaline articular cartilage for applications in arthritis disease modeling, drug screening, and regenerative medicine. However, the low efficiency and variability of hiPSC chondrogenesis has been a major hurdle for these applications. Recently, critical insights from developmental biology have elucidated the sequence of inductive and repressive signaling pathways needed for PSC lineage specification to a number of cell fates. By applying this signaling logic we sought to guide hiPSC differentiation in a step-wise fashion along a paraxial mesoderm lineage to chondroprogenitors (CPs) and ultimately chondrocyte-like cells. We further hypothesized that purification of CPs using a genome-engineered *COL2A1-GFP* knock-in reporter would improve chondrogenic differentiation and *in vitro* cartilaginous matrix production. We validated our step-wise differentiation approach in three well characterized hiPSC lines. Over the course of differentiation, we observed a gradual decrease in expression of the pluripotency markers *OCT4* and *NANOG* in each line. By day 2 of differentiation, we observed activation of paraxial mesoderm marker, *MSGN1* (750-fold,$p<0.05$), which returned to baseline levels by day 3. Differentiation to a chondroprogenitor population was evidenced by activation of *COL2A1* (331-fold,$p<0.05$) and *SOX9* (39-fold,$p<0.05$) at day 12. CPs self-aggregated to form pellets, which produced cartilaginous matrix rich in glycosaminoglycan and type II collagen when stimulated with TGF-β3. To further improve homogeneity and production of cartilaginous matrix, we sought to purify CPs by engineering a *COL2A1-GFP* knock-in reporter line with CRISPR-Cas9. When applied to our differentiation protocol, we observed the generation of distinct GFP^{low} and GFP^{high} populations at day 21. Enrichment of chondrogenic marker expression *COL2A1* (6.4-fold,$p<0.05$), *SOX9* (5.2-fold,$p<0.05$), and *ACAN* (6.6-fold,$p<0.05$) was observed in the GFP^{high} population compared to unsorted controls. Tissue generated from GFP^{high} cells exhibited more homogeneous distribution of glycosaminoglycan (GAG) void of fibrous tissue as evaluated by Safranin-O stain compared to unsorted controls. Biochemical analysis quantitatively confirmed the increased GAG content of tissue derived from GFP^{high} cells. Compared to unsorted cells, cartilaginous matrix produced by GFP^{high} cells exhibited a more hyaline phenotype, enriched for type II and VI collagens and depleted of type I collagen. Here, we describe an efficient, step-wise protocol for *in vitro* chondrogenesis through the paraxial mesodermal lineage that is consistent among multiple hiPSC lines. Further purification of CPs improves the production and quality of hyaline cartilage-like tissue. The efficient generation and purification of hiPSC-derived CPs may be valuable for both articular cartilage tissue engineering and patient-specific arthritis disease modeling.

Oligonucleotide Therapeutics

72. Systemic Delivery of PPMO Results in Widespread Muscle Delivery and Efficacy in Mice and Non-Human Primates for the Treatment of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a neuromuscular X-linked recessive disease most commonly caused by whole exon deletion mutations in the *DMD* gene that disrupt the mRNA reading frame and prevent dystrophin protein production. One strategy to treat DMD is to use antisense oligonucleotides to alter the splicing pattern (i.e., exon skipping) in order to restore the mRNA reading frame and enable production of an internally shortened dystrophin protein. A PPMO (peptide-conjugated phosphorodiamidate morpholino oligomer) compound that contains a cell-penetrating peptide (CPP) was injected into *mdx* mice and cynomolgus monkeys to evaluate potential improvement in dystrophin production and muscle function. In *mdx* mice, PPMO treatment resulted in high levels of exon skipping and dystrophin protein in muscle. PPMO-treated *mdx* mice also produced significant improvements on the grip strength and rotarod tests in a dose-dependent manner compared to saline-treated *mdx* mice, with the highest levels of functional recovery correlating to 21-25% wild-type levels of dystrophin in skeletal muscle as measured by western blot. To determine if widespread muscle delivery and efficacy is achievable with the same CPP in large mammals, non-human primates (NHPs) were treated with SRP-5051, a PPMO consisting of the CPP conjugated to a human PMO sequence which promotes exon 51 skipping. NHPs received 4 weekly injections of SRP-5051 at 20, 40 or 80 mg/kg. Exon

skipping was observed in all muscle tissues analyzed at the three dose levels, apart from the heart at the lowest dose of 20 mg/kg. The highest levels of skipping were observed at 80 mg/kg, which produced 65.7-94.9%, 60.7%, and 43.9-66.6% exon 51 skipping in skeletal (quadriceps, diaphragm, biceps, deltoid), cardiac (heart), and smooth (duodenum, esophagus, aorta) muscles, respectively. Taken together, these results indicate that intravenous administration of conjugated CPP and PMO (PPMO) results in high levels of dystrophin production *in viv*o, and that SRP-5051 treatment in NHPs results in robust and widespread exon 51 skipping in muscle tissues that are highly affected in DMD.

73. Inhibition of Extracellular Histones in Sepsis-Induced Cardiovascular Function

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Sepsis is the leading cause of morbidity and mortality in noncoronary intensive care units in the western world. Septic patients often develop myocardial dysfunction, leading to a phenomenon known as myocardial depression in sepsis (MDIS). This is mediated by the release of histones into the extracellular space by dying cells and the release of neutrophil extracellular traps (NETs) from white blood cells. In this study, we have identified RNA oligonucleotides (aptamers) that bind with high affinity and specificity to those histones implicated in MDIS (histone H3 and H4) using Systemic Evolution of Ligands by Exponential Enrichment (SELEX) technology. The ability of aptamers to reverse histone-induce toxicity was evaluated in cells in culture, and in an animal model of sepsis (cecal ligation and puncture - CLP). To date, we have demonstrated that the aptamers can reverse histone-induced platelet aggregation and prevent cytotoxicity of human lung-derived endothelial and epithelial cells. In vivo studies have focused on developing and characterizing the CLP model of sepsis and evaluating the safety of the aptamer bio-drugs in mice. With regards to the CLP model, we have observed an increase in histone levels in serum of septic (CLP) mice but not sham control mice. Importantly, the increase of histones in circulation correlated with degree of sepsis severity in these mice. This was analogous to the increase in histone levels observed in serum of septic patients. Ongoing studies are evaluating the efficacy of the aptamers in the CLP model. In conclusion, we present robust preclinical data on a novel class of therapeutics against circulating histones that may be potentially effective in the treatment of septic patients with MDIS.

74. Rna Inhibitors of Nuclear Proteins Implicated in Multiple Organ Dysfunction Syndrome

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Development of multiple organ dysfunction/failure, known as multiple organ dysfunction syndrome (MODS), is the most important clinical event following infection or extensive tissue injury, as it directly correlates with morbidity and mortality. Following the initial cellular injury, the most abundant nuclear proteins, histones are released into the circulation causing further injury. Once in the circulation, histones interact with *1)* platelets, causing aggregation resulting in thrombi formation; *2)* toll-like receptors resulting in cytokine production; and *3)* cellular membranes, triggering high calcium influx that leads to cell death. These extracellular histone effects augment tissue and cellular injury leading to a positive feedback loop of histone release. Unfortunately, anti-histone therapies (e.g., heparin and toll-like-receptor blocking antibodies) have failed in clinical trials due to non-specific effects causing bleeding and systemic toxicity. Here, we describe a novel therapeutic strategy for MODS based on chemically-stabilized nucleic acid bio-drugs (aptamers). We have generated aptamers utilizing systematic evolution of ligands by exponential enrichment (SELEX) technology that selectively bind to the extracellular histones implicated in MODS, but not other proteins in serum. We demonstrate the efficacy of these histone-specific aptamers in human cells in culture (e.g. inhibition of histone-mediated platelet aggregation, IL-6 production, and calcium influx) and in a murine model of MODS. Given the many etiologies of MODS, these aptamers could have a significant impact on the treatment of numerous clinical conditions associated with multiple organ dysfunction/failure.

75. Targeted Delivery of miR-146a Mimic or Antisense Oligonucleotides as a Potential Therapeutic Approach to Modulate NF-κB Signaling in Cancer and Autoimmune Diseases

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NF-κB signaling plays central role regulating cancer cell survival and self-renewal as well as immune cell activity. There is accumulating evidence that miRNA expression is dysregulated in various cancers, including hematological malignancies. The microRNA-146a provides negative feedback inhibition of the NF-κB pathway and has been suggested implicated in autoimmune disease such as graft versus host disease (GVHD) and acute myeloid leukemia (AML). While it is a potential therapeutic target, the lack of efficient delivery of miRNA remains a challenge limiting clinical translation. We previously developed a strategy for targeted delivery of oligonucleotide therapeutics, such as siRNA, into human and mouse myeloid cells

and B cells, using partly or completely phophorothioated (PS) single-stranded oligodeoxynucleotides containing CpG motif (CpG ODN). The CpG-conjugates undergo rapid scavenger receptor (SR)-mediated endocytosis followed by a Toll-like receptor-9 (TLR9)-facilitated cytoplasmic release. Here, we demonstrate that similar strategy based on type-A CpG ODN (D19) can be employed for the delivery of functional miR146a mimics as well as anti-miR146a oligonucleotides (146AMO). Both CpG-miR146a mimic and CpG-146AMO conjugates, but not unconjugated miRNA or AMO, are quickly internalized by target human and mouse myeloid cells, including AML cells. We confirmed the functionality of both CpG-miR146a mimic and CpG-146AMO in myeloid cells *in vitro*. The CpG-miR146a mimic reduced protein levels of downstream targets, such as Irak1 and Traf6, thereby inhibiting NF-κB activity at the DNA binding and transcriptional levels. As expected, CpG-146AMO had an opposite effect and strongly stimulated NF-κB signaling. We further confirmed functional activity of CpG-miR146a mimic in miR-146a knock-out mice by demonstrating that intravenous injections of this oligonucleotide can reduce the myeloproliferation and cytokine response to bacterial endotoxin challenge. In addition, our initial studies showed that systemic administration of the CpG-miR146a mimic can ameliorate GVHD in wild-type mice after allogeneic bone-marrow transplantation. Finally, we verified in mouse model of acute myeloid leukemia (AML; *Cbfb/Myh11/Mpl*) that immunostimulatory properties of CpG-146AMO can reduce numbers of leukemic cells in various organs and prolong mice survival. Our further studies will dissect molecular and cellular underpinnings of CpG-miR-146a mimic and CpG-146AMO effects *in vivo.* We believe that further optimization of these oligonucleotide strategies will generate novel therapeutics for precise immunomodulation in treatment of cancer and autoinflammatory diseases.

76. Artificial microRNA Silences C9ORF72 Variants *In Vivo* and Decreases the Toxic Dipeptides in BAC Transgenic Mouse Model

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Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease that targets upper and lower motor neurons causing progressive muscle weakening. Respiratory failure is the common cause of death approximately 2-5 years after symptom onset. In 2011 an expanded hexanucleotide repeat G4C2 was found in chromosome 9 open reading frame 72(C9ORF72), since then it has been shown to be the major cause of familial ALS and frontotemporal dementia (FTD). Brain tissue samples from patients have shown that in the presence of the expansion, C9ORF72 mRNA is reduced and exhibit RNA aggregates complimentary to the expansion. In addition, it has been shown that this RNA is giving rise to di-peptide chains by repeat-associated non-ATG translation. These findings have led to multiple hypotheses on the pathogenesis of C9ORF72: 1) Haploinsufficiency, where decreased levels of mRNA lead to insufficient gene product. 2) An RNA gain-of-function, where the expanded RNA foci sequester RNA binding and/or splicing proteins. 3) Non- ATG translation from the repeat expansion which generates inclusions of toxic poly-dipeptide proteins. Due to lack of treatments for this disease, we have pursued an AAV-RNAi dependent gene therapy approach, using an artificial microRNA packaged in a recombinant adeno-associated virus (rAAV) serotype 9. In order to further validate our *in vitro* results, we moved forward to *in vivo* experiments using a transgenic mouse model expressing a full human C9ORF72 gene, with a ~100-1000 repeat hexanucleotide expansion. This mouse model recapitulates the major histopathological features seen in human ALS/FTD patients such as: lower levels of human C9ORF72 mRNA, RNA nuclear aggregates, and the Non- ATG translation products. Neonatal mice with this expanded human allele where injected via an intracranial ventricular (ICV) route, additionally adult mice were injected with the rAAV-RNAi vectors via bilateral striatal injections. Our results suggest that AAV9-mediated microRNA silencing not only reduced the mRNA levels of C9ORF72 by 50% but also the short 222 aa. (24kDa) and long (54kDa) C9ORF72 protein. Importantly it also leads to a decreased production of the expansion derived toxic dipeptides. These encouraging results warrant the continued testing of this this treatment as a therapeutic option for ALS patients with a C9ORF72.

77. RNA-Based Combination Therapy for Diabetic Wound Healing

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The incidence of type 2 diabetes and chronic foot ulcers is increasing rapidly. In diabetic wounds, the normal response to hypoxia is impaired and many cellular processes involved in wound healing are hindered. Hypoxia Induced Factor-1α (HIF-1α) is a master regulator of the hypoxia response, activating multiple factors that promote cellular motility and proliferation, new vessel formation, and re-epithelialization—all important to the wound healing process. PHD2 (Prolyl Hydroxylase Domain-containing protein 2) regulates HIF-1α activity by targeting it for degradation under normoxia. HIF-1α also upregulates microRNA miR-210, which in turn regulates proteins involved in cell cycle control, DNA repair and mitochondrial respiration in ways that are antagonistic to wound repair. We have identified a highly potent short synthetic hairpin RNA (sshRNA) that inhibits expression of PHD2 and an antisense oligonucleotide (antimiR) that inhibits miR-210. Both oligonucleotides were chemically modified for improved biostability and to mitigate potential immunostimulatory effects. Using the sshRNA to silence PHD2 transcripts stabilizes HIF-1α and, in combination with the antimiR targeting miR-210, increases proliferation and migration of keratinocytes *in vitro*. To assess activity and delivery in an impaired wound healing model in diabetic mice, PHD2-targeting sshRNAs and miR-210 antimiRs both alone and in combination were formulated for local delivery to wounds using layer-by-layer (LbL) technology. LbL nanofabrication was applied to incorporate sshRNA into a thin polymer

coating on a Tegaderm mesh. This coating gradually degrades under physiological conditions, releasing sshRNA and antimiR for sustained cellular uptake. Formulated treatments were applied directly to splinted full-thickness excisional wounds in db/db mice. Cellular uptake was confirmed using fluorescent sshRNA. Wounds treated with a single application of PHD2 sshRNA or antimiR-210 closed 4 days faster than untreated wounds, and wounds treated with both oligonucleotides closed on average 4.75 days faster. Markers for neovascularization and cell proliferation (CD31 and Ki67, respectively) were increased in the wound area following treatment, and VEGF was increased in sshRNA-treated wounds. Our results suggest that silencing of PHD2 and miR-210 either together or separately by localized delivery of sshRNAs and antimiRs is a promising approach for the treatment of chronic wounds and has the potential for rapid clinical translation.

78. Modulation of Ldlr and Cholesterol by Transcriptional Silencing of the Long Non-Coding Rna Bm450697

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The Low Density Lipoprotein receptor (LDLR) is a cell surface expressed protein that binds and internalizes low density lipoprotein (LDL), resulting in cholesterol being made available to the cell. A method to stably over-express LDLR could result in an increased removal of LDL from the blood and subsequent lowering of cholesterol. Previous studies have uncovered an antisense non-coding transcript that overlaps the LDLR promoter, EST BM450697. We investigated the role of this transcript on LDLR gene expression and screened several siRNAs targeted towards either BM450697 or its promoter in Hep 3B and Hep G2 hepatocellular carcinoma cell lines. We show here that the overexpression of BM450697 decreases LDLR mRNA expression in a dose dependent manner. Using CHIRP analysis we found that BM450697 is enriched at particular loci in the promotor of LDLR. siRNA targeted transcriptional suppression of lncRNA BM450697, results in a concomitant increase in LDLR mRNA expression. Antisense oligonucleotides of the candidate siRNAs confirmed that the siRNAs target the lncRNA, increasing LDLR mRNA and protein expression. Pooling of candidate siRNAs result in an additive effect, increasing LDLR expression and uptake of LDL. Lastly, both single stranded RNA and siRNA GalNAC conjugates were experimentally validated for functional alteration of BM540697 and LDLR. The results of the observations presented here suggest that small RNAs can functionally activate LDLR expression by the targeted inhibition of the LDLR regulatory lncRNA, BM450697. Notably, these small RNAs are amendable to liver targeted conjugations, such as GalNAC and target the repression of BM450697 in an epigenetic manner, suggesting that long-term activation of LDLR might be feasible by stable silencing of BM450697.

AAV Vectors I

79. Chem-AAV: Chemically Modified AAV for Targeting Gene Delivery

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Recombinant AAV vectors are now becoming therapeutic products. However, several clinical trials using AAV have shown critical limitations: (i) high doses are usually required to achieve therapeutic effect; (ii) broad biodistribution to non-target tissues; (iii) loss of efficacy in the presence of pre-existing neutralizing antibodies. In an attempt to overcome these barriers, here we have used organic chemistry to improve the "specific activity" and the "therapeutic index" of rAAVs. For this purpose, *N*-acetylgalactosamine ligand (GalNAc), with an isothiocyanate (-NCS) coupling function, was synthesized since GalNAc recognizes the asialoglycoprotein receptor (ASPGR) present at the surface of hepatocytes. The GalNAc-NCS was grafted on AAV2 particle by reacting with amino groups present at the surface of the capsid. Multiple analytical tools were used to show, unambiguously, a covalent coupling of the GalNAc molecule to the surface of the capsid by formation of a thiourea function and not an adsorption of this molecule. Notably, we also demonstrated that it is possible to modulate the number of GalNAc ligands grafted to the AAV particles according to the experimental conditions used in the chemical reaction (i.e. number of GalNAc Equivalents (Eq)/ AAV particles). Indeed, Dot and Western blot analysis showed higher amounts of GalNAc molecules covalently coupled on the surface of the AAV2 capsid by using increasing amounts of ligands (3e5 vs 3e6 Eq). Importantly, GalNAc-AAV2 particles were more efficient in transducing mice primary hepatocytes in culture than wild-type AAV2 and the efficacy was boosted by using increasing amounts of ligands. Based on these encouraging results, we decided to use our chemical modification technology to increase the specific targeting of AAV towards the retina. To this end, a mannose ligand (Man) with a -NCS coupling function was synthesized and coupled to the AAV2 because mannose receptors are present in the retina. Dot and Western blot analysis showed increasing amounts of mannose molecules covalently coupled on the surface of the AAV2 capsid by using increasing amounts of ligands (3e5 vs 3e6 Eq). Importantly, Mannose-AAV2 particles were more efficient in transducing the retina than non-modified AAV2 upon subretinal injection of rats. Indeed, the expression kinetics, GFP diffusion and intensity was higher in eyes injected with mannose-AAV2 with 3e6 Eq. In summary, our data shows the possibility to modulate at will the quantity and the type of ligands at the surface of the AAV particles and opens novel possibilities for AAV-mediated targeted gene transfer.

80. Generation of Light-Producing Somatic-Transgenic Mice Using Adeno-Associated Virus Vector

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We have previously designed a library of lentiviral vectors to generate somatic-transgenic rodents to monitor signalling pathways in diseased organs using whole-body bioluminescence imaging. We have shown that this allows for data collection in conscious, freely moving rodents. We have now expanded this technology to include the use of adeno-associated viral vectors for the generation of somatic-transgenic rodents. We first explored biodistribution by assessing GFP expression after neonatal intravenous delivery of AAV8. We observed widespread gene expression in tissues including the central and peripheral nervous system, liver, kidney and skeletal muscle. Next, we selected a constitutive SFFV promoter and NFκB binding sequence from our existing library of more than twenty transcription factor binding elements for bioluminescence and biosensor evaluation. An intravenous injection of AAV8 containing firefly luciferase and eGFP under transcriptional control of either element resulted in strong and persistent widespread luciferase expression throughout the body of the animal (Figure 1). A single dose of LPS induced a 10-fold increase in whole-body luciferase expression in the AAV8 NFκB mice (Figure 2). Immunohistochemistry of brain sections revealed GFP expression in cells of astrocytic and neuronal morphology following intravenous injection of AAV8 NFκB. Importantly, we were able to show that whole-body bioluminescence persisted up to 240 days of age. To further restrict biosensor activity to the CNS, we performed intracerebroventricular injection of each vector. We observed greater restriction of bioluminescence to the head and spine with both vectors. Immunohistochemistry revealed strongest expression in cells of neuronal morphology. LPS administration stimulated a 4-fold increase over baseline bioluminescence. We have validated the biosensors toolkit by using a NFκB response element and reveal its potential use to monitor this signalling pathway in a non-invasive manner in a model of LPS-induced inflammation, systemically and also in the central and peripheral nervous systems. This technology complements existing germline transgenic models and may also be applicable to other rodent disease models with the use of different response elements.

Figure 1

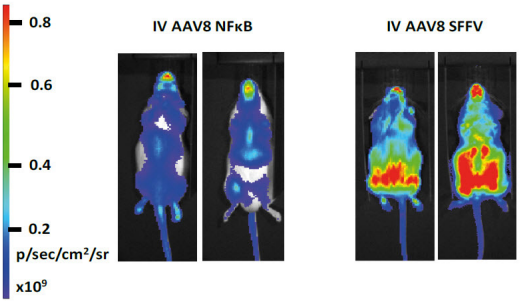
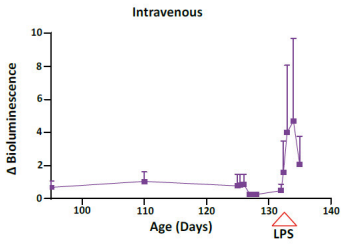


Figure 2



81. Disruption of the Heparin-Binding Site and Insertion of the PHB.P Peptide in AAV-DJ Improve Transduction of the Central Nervous System

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The development of targeted gene therapy in the central nervous system (CNS) is important for advancing new therapeutic approaches to treat neurological disorders. The non-pathogenic adeno-associated virus (AAV) vector has emerged with high potential for *in vivo* gene delivery. Recent discovery by Deverman *et al.* (Nature biotech., 2016) showed that an insertion of PHP.B, a seven amino acid peptide, into the capsid of AAV9 greatly improved the ability of the virus to cross mouse blood brain barrier (BBB). This allowed more efficient and global transduction of the CNS after intravenous injection. In this current study, we explored if insertion of the PHP.B peptide into a different AAV capsid would enhance its CNS transduction profile. We inserted the PHP.B peptide into the AAV-DJ capsid and a heparin-binding domain mutated AAV-DJ/8 capsid. When tested in a human *in vitro* trans-well BBB model, we found that both disruption of the heparin-binding site and insertion of the PHP.B peptide increased the permeability of AAV-DJ capsid. However, neither AAV-DJ variant was more permeable than AAV9 or AAV9-PHP.B. Next, we performed intravenous injection of the AAV-DJ variants containing a CAG promoter driven luciferase reporter into mice (n=5 per group). Bioluminescence imaging showed that the AAV-DJ/8 and AAV-DJ/8-PH.P had higher rates of transduction of the brain region compared to AAV-DJ. At three days post injection, AAV-DJ/8 resulted in a 10-times higher signal compared to AAV-DJ, while the AAV-DJ/8-PHB.P signal was 20-times higher than AAV-DJ. During the next three weeks, the transduction rate of AAV-DJ/8 and AAV-DJ/8-PHP.B were similar, expressing ~2.5-fold more than AAV-DJ. Further validation was established by quantifying luciferase activity in harvested tissues three weeks post vector injection. We found the transgene in activity various

CNS regions in AAV-DJ/8-PHP.B injected mice were similar to AAV9, both of which showed much higher activity than AAV-DJ/8. These studies show that disruption of the heparin binding site and insertion of the PHP.B peptide enhances the BBB penetration of AAV serotypes other than just AAV9.

82. Screening of Barcoded Capsid Shuffled AAV Libraries Results in the Selection of Capsids with Enhanced Transduction Efficiency for Human Islets

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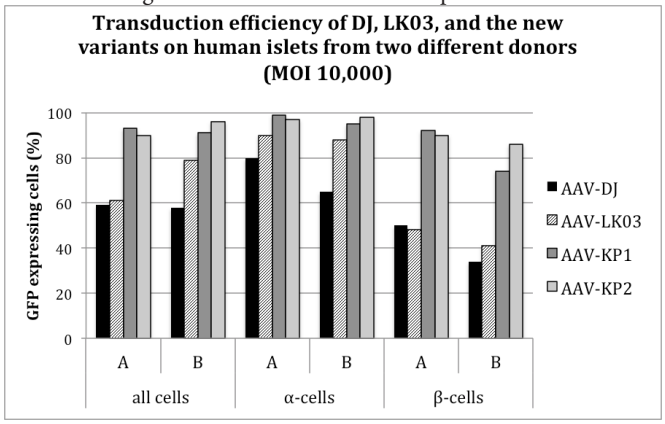
Gene therapy for the islets of Langerhans is a promising approach for the treatment of diabetes, particularly type 1. Recent data suggest that conversion of glucagon producing α- into insulin producing β-cells can be achieved by overexpression and / or repression of certain transcription factors. Moreover, for the purpose of islet transplantation, *ex vivo* gene therapy with protective factors (anti-apoptotic, pro-angiogenic) may improve graft survival and function. Adeno-associated virus (AAV) has been shown to be a very safe and effective vehicle for gene delivery into various human tissues. However, AAV gene therapy for islets is hampered by a lack of AAV serotypes that transduce those cells with high efficacy and specificity. In order to identify new AAV serotypes with improved tropism for human islets, we infected human islets with two replication competent highly complex capsid shuffled AAV libraries. The libraries were designed to contain unique barcode sequences downstream of the cap gene, facilitating high-throughput tracking of variant enrichment by deep sequencing of the barcodes. The libraries were subjected to three consecutive rounds of passaging on human islets in presence of human adenovirus 5 to replicate AAV. The most enriched capsid variants were used to package a GFP expression vector and transduction efficiency of dissociated human islets was analysed by flow cytometry. We identified two capsid variants (AAV-KP1 and AAV-KP2) that at a multiplicity of infection (MOI) of 1,000 transduced dissociated islets with 6.5-fold or 5-fold higher efficiency when compared to AAV-DJ or AAV-LK03, respectively. AAV-DJ is a capsid variant that had been isolated after subjecting a capsid shuffled library to stringent selection on a human hepatoma cell line (Grimm *et al*., J.Virol. 2008). AAV-DJ has been described to transduce a broad range of cell types with high efficiency. AAV-LK03 was derived from an *in vivo* screen of a capsid shuffled library in mice with humanized livers (Lisowski *et al*., Nature 2014) and shows good transduction of human islets, particularly of α-cells (Galivo *et al*., unpublished). In order to determine if the new AAV variants exhibit preferential transduction of α- or β-cells, intact islets were transduced with an MOI of 10,000 and stained with endocrine pan-islet as well as α-cell specific surface antibodies prior to analysis by flow cytometry. At this saturating MOI, the new variants transduced an average of 85% of the β-cell population, while AAV-LK03 only transduced 44% of β-cells (see Figure). Transduction of α-cells was also improved for the both variants (97% *vs.* 89% for LK03). These numbers represent average values from two independent experiments using islets from different donors. These novel AAV capsid variants, capable of penetrating intact, undisocciated human islets represent a powerful gene therapy tool for functional genomics and the treatment of patients with diabetes.



83. A Screen Reveals Putative Non-AAVR Receptors Utilized by an Airway Efficient AAV

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Cystic fibrosis lung disease is a target for gene therapy due to its monogenecity and the accessibility of airways to treatment. Adeno-Associated Virus serotype 2 (AAV2) is among the preferred vectors for gene delivery. AAV2 is safe, and was recently FDA approved for the treatment of inherited retinal dystrophy; however, when aerosolized into the airways of people with cystic fibrosis, AAV2 is inefficient. We previously selected a novel serotype, AAV2/2.5T, from a library of AAV2 and AAV5 chimeras on human airway epithelia (HAE). Unlike AAV2 or AAV5, AAV2/2.5T is efficient and binds specifically to relatively abundant apical receptors on HAE. This led us to hypothesize that the Adeno-Associated Virus Receptor (AAVR) required by AAV2 and AAV5 was not expressed in apical HAE, where our novel vector can use an alternative receptor. Our earlier unpublished findings demonstrated that AAVR is not functional in the apical membrane of HAE, and that AAV2/2.5T can utilize, but does not require AAVR. Here we expand on those results by 1) providing microscopic evidence of AAVR in the basolateral membrane of HAE, 2) showing that overexpression of AAVR in HAE preferentially facilitates basolateral infection, and 3) identifying candidates for the alternative receptor used by AAV2/2.5T. The goals of this study were to further validate the localization of AAVR in HAE, and generate putative identities for the non-AAVR receptor utilized by AAV2/2.5T. On HAE, apical AAVR was undetectable by immunofluorescence, but was found in the basolateral membrane after adenovirus mediated overexpression. The use of anti-AAVR antibodies ($AAVR_{AB}$) to block infection revealed that AAVR functions on the basolateral but not the apical membrane. Further, overexpression of AAVR preferentially increased basolateral infection up to 650 times over apical infection. On HeLa cells, $AAVR_{AB}$ blocked AAV2 and AAV5 infection, but AAV2.5T was less susceptible. The three serotypes were equally susceptible to $AAVR_{AB}$ block on HEK293T cells. CRISPR knockout of AAVR produced HeLa cells permissive to AAV2/2.5T but refractory to infection by AAV2 and AAV5, and overexpression rescued the phenotype. These data suggest HAE lack apical AAVR and that AAV2/2.5T can utilize AAVR as well

as an alternative receptor found in HeLa but not HEK293T cells. This provided rationale for screening multiple immortalized cell lines. We overlaid cells with a gradient of $AAVR_{AB}$ and determined which lines remained permissive exclusively to AAV2/2.5T at high doses. We then subjected our results to bioinformatic analysis, subtracting the predicted membrane proteins in refractory cell lines from those predicted in AAV2/2.5T permissive cell lines. In this way we have formulated a list of strong candidates to be validated for a highly effective unknown receptor which can be exploited by an AAV for efficient gene therapy in human airways.

84. Analysis of AAV Capsid Protein Post Translational Modifications and Their Effects on AAV Transduction Potential

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The development of advanced analytical methods for characterization of AAV viral capsid protein (VPs), including direct LC/MS and LC/MS/MS, has facilitated the characterization of constituent viral capsid proteins of AAV vector therapeutics, including their sequences and post-translational modifications (PTMs) *(Jin et al., 2017*). Post-translational modifications (PTMs), such as N-terminal acetylation, deamidation, and phosphorylation are common means of controlling protein function. N-terminal acetylation is the most abundant PTM, and involves the irreversible transfer of an acetyl group from acetyl coenzyme A to the alpha amino group of the first amino acid residue of a protein. In contrast, deamidation is a nonenzymatic process, which requires the irreversible, spontaneous loss of the amide group of asparagine or glutamine to form aspartic acid or glutamic acid, respectively. The role of AAV capsid protein PTMs on AAV transduction potential was evaluated by generating AAV capsid variants, with changes in amino acid residues associated with key post translational modifications. The amino acid changes were generated in two serotypes including AAV2, with transduction assessments of the AAV variants performed in vitro in HuH7, HeLa and 293 cells, and in vivo with vitreal delivery to the mouse eye. We show that a potential deamidation site affects vector potency, while N-terminal acetylation provides a critical signal for effective AAV transduction. AAV capsid PTMs do not affect tropism, but rather exert their effects at a post cell entry level. Interestingly, we find that different AAV manufacturing processes may induce different levels of PTMs, and the potential impact of this difference on vector potency will be discussed in the context of producer cell line and triple transfection produced AAV vectors. This work further underscores the importance of establishing rigorous analytical methods to fully characterize AAV vector therapeutics to ensure consistency in their quality and by extension potency.

85. Translational Fidelity of Photoreceptor Specific AAV Capsids and Promoter Elements

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The efficacy of AAV gene therapy for inherited retinal degenerative diseases has now been established in both preclinical studies and clinical trials. Retinal degenerative diseases are caused by genetic mutations in a variety of retinal cell types consequently a universal AAV capsid may not be useful for the treatment of disparate retinal dystrophies. To guide the selection of an optimal AAV serotype for the treatment of rod photoreceptor mediated retinal dystrophies, such as Retinitis Pigmentosa, the efficacy of AAV5 and a novel variant AAV2HBKO were compared in the context of the mouse and NHP retina. The AAV2HBKO variant, was generated by targeted mutation of arginine 585 and 588 in the parental AAV2 capsid, both arginines are implicated in heparin sulphate binding and when changed to alanine generate a variant, AAV2HBKO, which is devoid of heparin sulphate binding (*Sullivan et al., 2018*). AAV5 and AAV2HBKO vectors were produced by packaging the same expression cassette consisting of EGFP driven by a ubiquitous promoter or a rod cell specific promoter AAV5 and AAV2HBKO, in conjunction with the ubiquitous promoter, were found to be effective, and comparable, at transducing both rod and cone photoreceptors as well as retinal pigmented epithelial cells, following subretinal injection into nondegenerate mouse eyes. In contrast, use of a a rod cell specific promoter, resulted in restriction of EGFP expression to rod photoreceptors, following subretinal delivery of either serotype to the mouse retina. A quantitative assessment of transgene expression in the mouse retina was performed using an EGFP specific ELISA, which confirmed that the EGFP expression was greatest from the ubiquitous promoter for both AAV5 and AAV2HBKO, with overall retinal expression trending lower with the rod cell specific promoter. AAV5 and AAV2HBKO, in the context of the rod cell specific promoter and EGFP transgene, were additionally tested in the cynomolgus monkey (*Macaca fascicularis)* retina, to determine if there was species specific tropism or promoter performance differences. A single sub retinal bleb, containing 1e11vg of either AAV5 or AAV2HBKO vector was introduced into the NHP retina, approximately 6 weeks post vector delivery retinas were analyzed by immunohistochemistry to confirm EGFP localization and determine the percentage of transduced cells. For both vector groups, the intensity of EGFP expression was comparable, and expression was observed in the outer nuclear layer, specifically in the rod photoreceptors, as determined by co-localization of rhodopsin protein staining.. Additionally, there was a slight difference in the pattern of transduction between the two vectors, with the AAV5 vector showing transduction patterns consistent to the edge of the bleb, in contrast the AAV2HBKO vector which showed transduction patterns that extended beyond the edge of the bleb. The latter finding was also supported by in vivo scanning laser ophthalmoscopy, cSLO, fundus imaging. In conclusion the performance of both AAV5 and AAV2HBKO in the context of the rod specific, human rhodopsin

promoter is comparable for both the mouse and NHP retinae, and the results presented here should help inform AAV serotype and promoter selection for basic and translational retinal research.

86. A Self-Deleting AAV-CRISPR System for In Vivo Genome Editing

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Significance/Background: Adeno-Associated Viral (AAV) vectors can deliver CRISPR/Cas9 components with high efficiency, resulting in permanent modification of disease relevant genes *in vivo*. However, there are safety concerns due to persistent expression of Cas9, which must be addressed before this technology can be translated into clinical use. In particular, off-target mutagenesis is dependent on the level and duration of Cas9 expression. Further, pre-existing immunity to the Cas9 nuclease has been observed in humans, which could result in elimination of edited cells. Methods are needed to achieve efficient genome editing *in vivo* while avoiding persistent expression of the bacterial Cas9 nuclease. We *hypothesized* that Cas9 could be used to eliminate its own expression *in vivo*, through targeted destruction of recombinant AAV episomes, while still achieving high on-target editing efficiency. **Methods:** Recombinant AAV vectors based on serotype 8 were constructed to express the Cas9 ortholog from *Staphylococcus aureus* (SaCas9) (AAV-CRISPR), driven by a small liver-specific promoter. Guide RNAs (gRNAs) targeting respectively endogenous murine genes (*Apob, Mttp*), green fluorescent protein (GFP), or SaCas9 itself were driven by the U6 promoter, either upstream on the same AAV8 construct, or in a co-delivered AAV8 vector with a GFP expression cassette. C57BL6/J mice were treated with a subset of these AAV8 vectors for liver-directed genome editing, and followed for 1-6 weeks. Endogenous and AAV episomal gene editing rates in mouse livers were determined through next generation sequencing. GFP, ApoB, and SaCas9 protein levels were measured by western blotting. **Results:** We first tested whether AAV-CRISPR could disrupt a co-expressed GFP transgene from AAV episomes in mouse liver. An AAV vector encoding both SaCas9 and a GFP targeting gRNA was able to efficiently eliminate GFP protein expression from a co-delivered AAV vector. Episomal AAV-GFP editing rates increased in a linear fashion over time, reaching a maximum of 82% Indel rate at 6 weeks after delivery. GFP protein was significantly reduced beginning at 2 weeks, and virtually undetectable within 3-4 weeks. We next constructed a self-deleting AAV-CRISPR system with a gRNA targeting SaCas9 itself. AAV delivery of SaCas9 with a gRNA targeting an endogenous gene (*Apob*) resulted in near complete editing within 10-14 days. Co-delivery of a self-deleting gRNA targeting SaCas9 substantially reduced SaCas9 protein levels with slower kinetics (~4-6 weeks). Interestingly, on-target editing efficiency for the endogenous gene was not adversely impacted by the self-deleting AAV-CRISPR system. **Summary/Conclusions:** We report a self-inactivating AAV-CRISPR system that is able to reduce Cas9 protein expression *in vivo,* while maintaining high endogenous on-target editing. This approach to control the duration of Cas9 expression *in vivo* will accelerate clinical translation of liver-directed genome editing for treating human diseases.

87. Identification of New Muscle-Tropic Adeno-Associated Virus (AAV) Capsids for Treatment of Rare Hereditary Muscular Disorders

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Myotubular myopathy (MTM) and glycogen storage disorder type II (GSDII, Pompe disease) are rare hereditary muscle diseases which can provoke significant morbidity and mortality due to skeletal muscle, cardiac and/or diaphragm dysfunction, and for which effective cures are still lacking. We aim to develop novel AAV vectors that will mediate robust delivery of therapeutic genes to all the affected muscle types in MTM or GSDII patients following minimally invasive, peripheral vector administration, ideally even in the presence of neutralizing anti-AAV antibodies. To this end, we used DNA family shuffling technology to create libraries of chimeric capsids composed of AAV serotypes with high efficiency in muscle tissues, AAV1, 6, 8 and 9. In one library, we also included AAVpo.1 based on prior evidence by us and others that this serotype robustly transduces especially skeletal muscle from intravenous delivery. We then subjected these libraries to multiple rounds of direct *in vivo* selection in adult C57BL/6 mice, composed of tail vein injection of each library and, one week later, PCR-based rescue of enriched AAV capsid genes in skeletal muscle, heart and diaphragm, followed by subcloning and preparation of secondary libraries for the next selection cycle. Interestingly, we observed a striking enrichment of AAV9-derived DNA sequences in the C terminus of most clones in all shuffled libraries that was already apparent after two selection rounds. In contrast, the N termini of these clones were composed of a mixture of all parental AAV serotypes, with no distinct accumulation or depletion of particular viral isolates. The only exception was AAVpo.1 which was rapidly lost during *in vivo* selection, albeit we could independently confirm the good performance of this serotype in muscle tissue. Thus, a most likely explanation is the low homology of the AAVpo.1 capsid gene and proteins with those of all other parental viruses in the libraries, implying that inclusion of AAVpo.1 has impaired viral particle stability and/or functionality. Next, we selected 33 chimeric clones from all libraries and re-assessed their *in vivo* efficiency and specificity using a new combinatorial DNA/RNA barcoding, next-generation sequencing and qPCR strategy. As benchmarks, we included wild-type AAV9 as a gold standard for muscle gene transfer from peripheral delivery, as well as three peptide-modified capsids including one that we have recently recognized as very potent in another screen by our group. Notably, this allowed us to identify four shuffled AAV capsids that show a markedly improved tropism in muscle tissues, especially diaphragm, heart and *quadriceps femoris*, and

a relative detargeting from the liver. We anticipate these capsids, when combined to muscle-specific promoters, to have a great potential for gene therapies of rare muscular disorders.

88. The Assembly-Activating Protein is Pleiotropic in AAV Assembly

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The ability to produce sufficient quantities of viral vector for gene therapy remains a bottleneck to the growth of the field. We seek mechanistic insight on adeno-associated virus assembly to ultimately optimize AAV reagents and processes for improved manufacturing. Previously, we demonstrated the Assembly-Activating Protein (AAP) functions both to stabilize the AAV viral capsid protein (VP) and to promote VP-VP interactions to nucleate capsid assembly. Through a novel phenotype-to-phylogeny mapping strategy that employs 12 natural serotypes and a set of 9 putative evolutionary intermediate AAV capsids (AncAAVs) we identified key residues involved in VP-VP interactions, that when strengthened reduce dependency on AAP. Moreover, many AAVs assemble into infectious particles without the need for the C-terminal two-thirds of AAP (AAPC), previously thought to be essential for assembly. Here, we show a variable ability for AAP of AAV2 to rescue heterologous AAV and AncAAV assembly. Using phenotype-to-phylogeny mapping, we harness these phenotypes to identify a candidate AAP binding site on VP's beta barrel. Preliminary co-immunoprecipitation experiments suggest this site is indeed important for VP/AAP interaction. Additionally, mutations targeting a patch of charged amino acids at this site abolish capsid assembly, yet have variable effects on VP protein levels depending on the identity of the introduced mutations. We demonstrate that VP is subject to both proteasomal and lysosomal/autophagosomal degradation. AAP-dependent serotypes, such as AAV8, appear to be preferentially degraded by the proteasome, and to some extent this may be Ubiquitin-independent, whereas AAPC-independent AAV3 is more susceptible to lysosomal/autophagosomal degradation. We explore motifs on VP that potentially target VPs for degradation, located near the putative AAP binding site and at the trimer interface, and demonstrate that rescuing VP protein by blocking degradation is not sufficient to rescue capsid assembly in the absence of AAP. Structural studies by cryo-EM reveal that a stretch of ordered N-terminal VP3 residues adjacent to the putative AAP binding site becomes disordered in capsids assembled without AAPC, suggesting flexibility in its absence. Taken together, our findings suggest the interaction of AAP with VP proteins is multifunctional: to shield residues or motifs that target VPs for degradation, and to aide VP oligomerization into ordered sub-assemblies, shielding additional such residues and increasing assembly efficiency by influencing secondary, tertiary and/or quaternary structure, in addition to the essential trafficking functions shown previously by others.

89. AAV-S1 Integration is Not Essential for High-Yield 293-Based AAV Producer Cell Line

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With the recent FDA approval of the first-in-class gene therapy drug voretigene neparvovec-rzyl (Luxturna™), AAV (Adeno-associated viral) vectors have repeatedly demonstrated their efficacy and safety in delivering therapeutic genes to treat previously incurable diseases. However, it remains a daunting task to produce high quality and large quantities of AAV. Previously, our laboratory pioneered the development of a high titer HEK-293 cell-based AAV2 packaging cell line. In this study, we investigated whether a high yield AAV9 producer cell line could be established by targeted delivery of AAV packaging plasmid into the AAVS1, the natural AAV integration site, of the HEK-293 cells. The site-specific integration was achieved by utilizing the clustered, regularly interspaced, short palindromic repeat (CRISPR)-associated protein 9 nuclease (Cas9) technology. Three plasmids (the inducible packaging plasmid pSPG9-D(+)-GFP-Hyg, the guide RNA plasmid gRNA-AAVS1-T2, and the Cas9 plasmid pX330-CBh-hSpCas9) were co-transfected into the HEK-293 cells. Meanwhile, the control group with only one packaging plasmid transfection without the CRISPR-Cas9 system was also carried out. Stable cell clones were grown under the selection of Hygromycin. The numbers of Hygromycin resistant clones were roughly counted and we did not notice a significant difference between the groups with or without CRISPR. Individual clones were expanded and screened by production of AAV-D(+)-GFP using real-time PCR titration method. Out of 50 cell clones from CRISPR group, four cell lines demonstrated significant yield of AAV vector, and their screening tiers range from 7.8 x 10^8 to 1.7 x 10^10 vg/one-well of 6-well plate. Two cell lines demonstrated significant AAV yield from 20 cell clones in the control group without CRISPR and their yields were: 4.1 x 10^9 and 1.8 x 10^10 vg/one-well of 6-well plate. We further characterized copy numbers of the integrated packaging plasmid Rep gene for each cell line with real-time PCR. The copy numbers of Rep gene in cell lines from CRISPR groups ranged between 3 to 6 copies/cell, while the rep copy numbers in cell lines from control group were 9 and 16 copies /cell. After Ad-Cre infection, the rep gene copy numbers were amplified by 5 to 10 fold in all cell lines. We additionally utilized our previously published cell line, AAV2-145 GFP, as the control for copy number determination. The rep gene in AAV2-145-GFP was around 75, and was amplified to around 300 copies per cell after Ad-Cre infection. Taken together, our results indicate that AAV-S1 site integration is not essential for a high-yield AAV producer cell line. On the other hand, the copy number of integrated packaging plasmid and amplitude amplification of the Rep gene are the key determinants of the high-yield AAV producer cell line.

90. Isolation of Novel Adeno-Associated Viruses by Single Genome Amplification and Sequencing

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Adeno-associated viruses (AAVs) are single-stranded DNA parvoviruses that are non-pathogenic and weakly immunogenic, which make them effective candidates as vectors for gene therapy. We have knowledge of over 200 AAVs that have been isolated from various sources, primarily from primate tissues. Currently used clinical AAV vectors are effective as gene therapy vectors, but they have been shown to have restricted tissue tropism and there is variable pre-existing immunity to these serotypes in the human population. AAV genomes have been traditionally isolated from whole mammalian genomic DNA using basic PCR based methods: primers are used to detect conserved regions that flank the majority of the diverse VP1 (capsid) gene. The PCR products are then cloned and Sanger sequenced. Here, we demonstrate AAV-Single Genome Amplification (AAV-SGA), a technique used to accurately isolate individual AAV genomes from within a viral population. AAV amplicons are subjected to next-generation sequencing and reads were subsequently de novo assembled into long, contiguous AAV genomes using SPAdes (http://cab.spbu.ru/software/spades/). AAV-SGA and sequencing were used to isolate and characterize novel AAVs from rhesus macaque liver tissue samples. Viruses from clades A, D, E, and the primate outgroup clade were found. Vector packaging and transduction efficiencies of the novel AAVs were assayed.

91. Chimeric Capsid Proteins Impact Haploid Virus Production and Transduction

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AAV intracellular trafficking is a rate limiting step for transduction and a majority of AAV capsids remain trapped in a perinuclear region, never reaching the nucleus. Intracellular trafficking is influenced by the unique region of VP1, which contains a phospholipase domain and nuclear localization signals, making its incorporation into capsids critical to AAV transduction. Previous work from our laboratory has shown that making haploid AAV capsids by using the VPs from multiple serotypes prevents serotype-specific antibody binding. To follow up on this work, we pursued the hypothesis that haploid viruses can also increase transduction by utilizing the VP1 and VP3s from compatible serotypes. These capsids were produced by transfecting mutated cap plasmids into 293 cells along with luciferase vector and helper plasmids. Virus was purified using CsCl purification and injected into mice at $2x10^{10}$vg/mouse and luciferase expression was measured at 1 week post-injection. We found that AAVs composed of VP1 from serotypes 7, 8, 9, and rh10 and VP3 from AAV2 or AAV3 display a 2 to 7-fold increase in transduction across multiple tissue types, including liver, heart, and brain compared to AAV2-only and AAV3-only capsids. These tissues also had higher vector genome copy numbers in these tissues as measured by qRT-PCR, indicating that a minimal incorporation of non-cognate VP1 can influence receptor binding and viral entry. In addition, we created chimeric and haploid capsids with either AAV2 or AAV8 VP1 combined with AAV2 or AAV8 VP3. When these capsids were injected into mice, the AAV2 capsids composed of AAV8 VP1/2 and AAV2 VP3 had a 5-fold higher transduction than capsids composed solely of AAV2 VPs. Remarkably, capsids composed of VP1 with the N-terminus of AAV2 and the C-terminus of AAV8 paired with VP3 from AAV2 had a 50-fold increase in luciferase activity compared to capsids composed of AAV8 VP1 paired with AAV2 VP3. Given the same proportion of the capsid coming from AAV8 VP3, the difference lies in the VP1/2 region between AAV2 and AAV8, which may indicate a 'communication' between the VP1/2 of AAV2 with its cognate VP3 or, alternatively, a role for AAV2 VP2 in transduction. During the course of these studies, we also found that the incorporation of VP1 from different plasmid sources played a role in transduction. By transfecting an increasing ratio of AAV2 VP1-only plasmid with AAV2 VP3-only plasmid, we discovered a higher incorporation of VP1 into capsids when VP1 was provided on a plasmid separate from VP3. A possible explanation of this may be that competition for transcription on the same plasmid reduces the pool of VP1 available for capsid assembly. By providing the reading frames on separate plasmids, a larger amount of VP1 may be produced and results in a 2-fold increase in expression. Experiments to understand the mechanisms behind these findings are currently underway. Collectively, these studies offer insight into current AAV production strategies that can increase transduction across multiple tissue types.

92. rAAV SOX9 Gene Transfer Stimulates the Chondrogenic Differentiation Activities in Human Peripheral Blood Aspirates

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Introduction: Implantation of genetically modified peripheral blood aspirates may be a promising approach to treat focal cartilage lesions[1]. Here, we explored the effects of rAAV- SOX9 in human peripheral blood aspirates, allowing to durably enhance the chondrogenic differentiation activities in the samples. **Methods:** rAAV-lacZ carries the E. coli beta-galactosidase (lacZ) gene and rAAV-FLAG-hsox9 a human FLAG-tagged sox9 sequence, both controlled by the CMV-IE promoter/enhancer[2,3]. Peripheral blood was collected in the presence of hirudin from human donors were immediately transduced with rAAV (40µl) or let untreated and kept in chondrogenic medium for up to 21 days. Histological and immunohistochemical analyses were performed on paraffin-embedded sections of the constructs (5 µm) (toluidine blue staining; anti-SOX9 and anti-type-II/-I/-X collagen immunostaining)[2,3]. The proteoglycan contents in the aspirates were monitored by binding to dimethylmethylene blue dye and the DNA contents by Hoechst 33258 assay[2,3]. Total RNA was extracted and reverse transcription carried out for cDNA amplification via real-time RT-PCR with GAPDH as control for normalization[2,3]. Each condition was performed in duplicate in three independent experiments. A t-test was employed with $p \leq 0.05$ considered statistically significant. **Results and Conclusion:** Transgene (SOX9) expression was observed in rAAV SOX9-treated aspirates relative to control conditions over the

period of evaluation (Fig. 1). Enhanced chondrogenic differentiation was achieved in the aspirates transduced with rAAV SOX9 after 21 days as noted by stronger toluidine blue staining and type-II collagen immunostaining (Fig. 2). Application of rAAV SOX9 significantly increased the proteoglycan contents in the aspirates relative to control treatments (1.8-fold) while no significant effects were noted on the DNA contents (Table 1). Of further interest, immunoreactivity to type-I and -X collagen was less intense when rAAV SOX9 was provided to the aspirates (Fig. 2). These findings were corroborated by results of a real-time RT-PCR analysis showing enhanced chondrogenic differentiation with SOX9 relative to the control treatments (up to 2- and 1.7-fold higher COL2A and ACAN expression, respectively; $p \leq 0.001$) and reduced hypertrophic differentiation (up to 2.7-fold lower COL1A1 and COL10A1 expression, respectively; $p \leq 0.001$), probably resulting from increased levels of SOX9 expression (up to 5-fold difference; $p \leq 0.001$) (Fig. 3). Conclusion: These in vitro results show the potential of targeting human peripheral blood aspirates via therapeutic rAAV-sox9 transduction as a novel, convenient tool to treat articular cartilage defects. **References:** [1] Frisch *et al.*, *Curr Stem Cell Res Ther* **2015**, 10:121; [2] Frisch *et al.*, *Stem Cells Transl Med* **2017**, 6:249; [3] Venkatesan *et al.*, *Stem Cell Res Ther* **2012**, 3:22. ACKNOWLEDGMENTS: Work supported by the German Research Society.

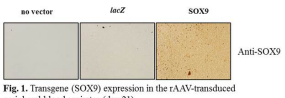

Fig. 1. Transgene (SOX9) expression in the rAAV-transduced peripheral blood aspirates (day 21).

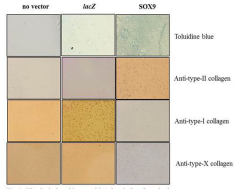

Fig. 2. Histological and immunohistochemical analyses in the rAAV-transduced peripheral blood aspirates (day 21).

Table 1. Biochemical analyses (day 21).

Treatment	PGs/total proteins (µg/µg)	DNA/total proteins (ng/µg)	PGs/DNA (µg/ng)
no vector	0.113 (0.2)	0.023 (0.1)	4.9 (0.2)
lacZ	0.137 (0.3)	0.018 (0.1)	7.6 (0.2)
SOX9	0.207 (0.1)$^{\#,*}$	0.010 (0.2)	20.7 (0.1)$^{\#,*}$

Data are given as mean (SD). PGs: proteoglycans. Statistically significant compared with #no vector and *lacZ.

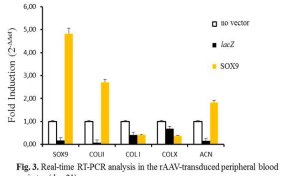

Fig. 3. Real-time RT-PCR analysis in the rAAV-transduced peripheral blood aspirates (day 21).

93. Sequential Bilateral Intravitreal Injection of GS010 (rAAV2/2-*ND4*) Separated by a 15-Day or a 3-Month Interval in Non-Human Primates: Good Safety and Immunogenicity Profile

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Leber hereditary optic neuropathy (LHON) is due in ~70% of all cases to the G11778 *ND4* mitochondrial DNA point mutation leading to central vision loss. GS010, a rAAV2/2-*ND4* gene therapy, is developed to treat *ND4* LHON patients via allotopic expression of human wt-ND4 protein in mitochondria of retinal ganglion cells. Given that LHON is a sequential bilateral disease, we are developing a bilateral clinical strategy with GS010. A GLP toxicity, biodistribution and immunogenicity study was conducted in Cynomolgus monkeys to cover a potential sequential clinical use. Eighteen male monkeys received intravitreal injection of vehicle or GS010 (4.3×10^{10} vg/eye, i.e. NOAEL from the single dose toxicity study) in the right eye at Day 1, and in the left eye either at Day 15 or at Month 3 (*i.e.* Day 92). After the second eye injection, animals were observed for 3 months. There were no unscheduled deaths, no effects on mean body weight and no GS010-related clinical signs. Ophthalmological examinations did not reveal any GS010 treatment-related findings. At necropsy, no organ weight differences, no macroscopic changes and microscopic findings especially in the eye or brain (thalamus) were recorded related to GS010 administration. A minimal and non-adverse decrease in the germinal center development of spleen was observed in primates treated 15-day apart. Since this change was not recorded in animals treated 3-month apart and examined at Month 6, the recovery was considered to be complete. Integration site analysis of GS010 within host cell genomic DNA did not reveal any indications for potential side effects, neither clonal outgrowth nor preferred integrations in oncogenes and indicate a low frequent level of integration within the retina samples. No integration was detected in spleen samples. Regarding biodistribution, GS010 vector DNA was quantified in almost all ocular humors, anterior segment and retina as well as spleen samples. GS010 DNA was also sporadically quantified in blood, optic chiasma, lacrimal gland, optic nerve and auricular lymph node samples from some animals up to Month 6. GS010 mRNA was expressed in all retina samples but not in spleen (except in one sample out of 4). Increasing levels of anti-AAV2 neutralizing antibodies (NAbs) were found in serum of all GS010-treated animals from 15 days after first injection and remained present until end of the study. For aqueous humor samples, NAbs were not found in contralateral eyes up to 3 months after the first eye treatment. In 3-month apart treated animals, only one contralateral eye out of 4 was found positive 15 days after its injection. In conclusion, in absence of local and systemic toxicological effects and given the safe integration profile, GS010 NOAEL was confirmed to be $4.3\text{x}10^{10}$ vg/eye in this sequential bilateral administration study design. A classical anti-AAV2 NAb serum response was induced in treated animals. NAbs were also quantified in aqueous humor in injected eyes of animals treated 3 months apart, without inducing observable ocular manifestations. These results raise the possibility that up to 3 months delay between eye treatments, GS010 administration will be safe and well tolerated.

94. CRISPR-Cas9 Based Therapeutic Genome Editing to Treat Age-Related Macular Degeneration

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Several CRISPR-Cas9 orthologues have been used for genome editing. Here, we present the smallest Cas9 orthologue characterized to date, derived from Campylobacter jejuni (CjCas9), for efficient genome editing in vivo. After determining protospacer-adjacent motif (PAM) sequences and optimizing single-guide RNA (sgRNA) length, we package the CjCas9 gene, its sgRNA sequence, and a marker gene in an all-in-one adeno-associated virus (AAV) vector and produce the resulting virus at a high titer. CjCas9 is highly specific, cleaving only a limited number of sites in the human or mouse genome. CjCas9, delivered via AAV, induces targeted mutations at high frequencies in

retinal pigment epithelium (RPE) cells. Furthermore, CjCas9 targeted to the Vegfa or Hif1a gene in RPE cells reduces the size of laser-induced choroidal neovascularization, suggesting that in vivo genome editing with CjCas9 is a new option for the treatment of age-related macular degeneration.

95. A Hybrid Phagemid-Derived Vector for Systemic Targeted Cancer Gene Therapy and Recombinant Virus Production

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Introduction: Gene therapy has the potential to provide curative treatment for incurable diseases such as cancer. Despite its appeal, an insurmountable problem is the limited number of efficient, safe, and specific vectors for clinical translation. In trying to preserve the advantages and overcome the pitfalls of eukaryotic and prokaryotic viruses, we have engineered a targeted hybrid phagemid vector that possesses the genetic characteristics of recombinant adeno-associated virus (rAAV), termed Phagemid-AAV (PAAV). In doing so, we are able to minimize the particle size of the vector and dramatically improve gene transfer efficiency. We also provide evidence to suggest that the PAAV could be adapted to replace conventional co-transfection systems for rAAV production. **Method:** The PAAV genome was designed and constructed using elements from commercial rAAV2 and phage display vectors. Functional vectors were produced, carrying a cyclic RGD4C ligand fused to its minor coat protein for tumour targeting. in vitro characterization of PAAV was done by transducing HEK293 and U87 cell lines with 1 million transducing units (TU) of virus per cell; the PAAV was evaluated against a hybrid AAV/phage virus with its genome fully intact. Vector internalization was monitored by staining phage capsid in permeablised cells, and gene expression was evaluated using GFP and luciferase activity. The PAAV was also physically characterized using transmission electron microscopy. To assess whether rAAV could be produced in 293 cells when transduced with phagemids derived from plasmids used in conventional transfection systems for rAAV expression, we performed quantitative-PCR using primers specific to the Inverted Terminal Repeat sequences (ITRs) to determine the gene copies of rAAV in the cell lysate. **Results:** The PAAV system yields up to two orders of magnitude higher number of functional particles per production round (average titre c.1e^{11}TU/µl) compared to the control virus (average titre c.1e^{9}TU/µl). The mean particle length of PAAV vectors was found to be 729.95±10.2nm, which is significantly smaller (c.50%) than the control ($p<0.01$). Furthermore, the internalization efficiency of PAAV vectors was significantly greater at c.1.6 times higher than the control, which subsequently translates in to GFP expression efficiency (7.7±0.65% GFP-positive cells, 2.4-fold higher than respective controls, $p<0.01$) and luciferase activity (up to 15-fold higher at day-3 post-transduction, $p<0.01$). In probing rAAV replication, it was found that the mean ITR gene copies (GC) per cell was 6.86±0.83 thousand GC/cell, which is over 3-fold higher than the number of ITRs found in the control condition (PAAV-transduced only, $p<0.05$). **Conclusion:** Our preliminary results suggest that the PAAV is a superior hybrid vector compared to currently existing phage or phagemid constructs designed for mammalian cell transduction. While it relishes the benefits of eukaryotic viral gene expression, the vector is easily produced at high titers and can easily be re-targeted using phage display technology. Our data also suggests that replication of ITRs does indeed occur when PAAV is co-transduced with helper elements commonly used in transfection systems for rAAV expression. Taken together, the PAAV provides a novel and superior alternative to conventional viral systems, and may potentially provide a platform for recombinant virus or protein production.

96. In Vitro Characterization of Novel AAV-Spark100 and AAV-Spark200 Capsid Variants

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Introduction: Preliminary results from our ongoing phase 1/2 clinical study for the treatment of hemophilia B have demonstrated expression of coagulation factor IX in ten subjects. Following vector administration, transgene-derived factor IX coagulant activity prevented spontaneous bleeding and the need for exogenous infusions, with no serious adverse events and no inhibitors against FIX observed on cumulative follow-up of 492 weeks among all the participants (George et al, N Engl J Med 2017). Preliminary results, albeit with a smaller cohort size and a shorter follow-up period, of our ongoing phase 1/2 dose escalation trial of an investigational AAV-mediated gene therapy for hemophilia A have recently been reported (George et al, Blood. Suppl 1 2017). Hepatocyte transduction mediated by the two capsids utilized, AAV-Spark100 and AAV-Spark200 for the hemophilia B and A studies, respectively, appears to be remarkably efficient, as evidenced by the low vector doses used. **Objectives:** We aimed to further improve liver tropism of the AAV-Spark100 and AAV-Spark200 capsids by employing rational design approaches for capsid engineering. **Results:** We employed a rational design approach to develop novel capsids with improved liver tropism, focusing on two methods. First, we introduced a series of single amino acid substitutions, primarily within the AAV-Spark200 capsid. These included homologous or analogous mutations previously described in the context of other serotypes, such as lysine to arginine substitutions (e.g. K137R), in addition to novel mutations specific to the AAV-Spark200 capsid. To facilitate vector production, given the large number of different capsid plasmids generated (>100), vectors were produced at small scale using HEK293 cells in 6-well dishes and crude lysates were titered by qPCR and tested *in vitro* in Huh7 cells. Although most of the capsids containing single point mutations packaged successfully, these substitutions did not result in a significant increase in cell transduction, as measured by luciferase expression. Our second rational design approach utilized peptide insertions of 7-mer, 9-mer, 11-mer or 27-mer peptides placed strategically into specific regions of the AAV-Spark200 and AAV-Spark100 capsids. Remarkably, peptide insertions that exhibited stronger increases in vector potency were, for the most part, common between the two serotypes, suggesting a capsid-independent mechanism of action. We also sought to investigate whether adding

multiple copies of these potency-increasing peptides combinatorially inserted in tandem (2x or 3x) within the AAV-Spark100 capsid would further increase vector potency as compared to the single peptides. Of note, some of the most effective capsids containing double or triple tandem insertions showed 4-9 times better transduction rates in Huh7 cells, compared with the parental serotype. Capsid yields were variable, with a potential correlation between peptide length and packaging efficiency. The mechanism(s) underlying the observed increase in vector potency after peptide insertion remains to be elucidated but could potentially include increased cell entry, higher endosomal escape, decreased proteasomal degradation and/or increased nuclear entry. On the basis of these preliminary results, we have selected ten novel capsids to proceed to further investigation of *in vivo* hepatic transduction in mice. **Conclusion:** We have developed a series of AAV-Spark100 and AAV-Spark200 capsid variants showing increased tropism for liver cells in vitro compared with the parental serotypes. These results in cell culture warrant further investigation of the potency of these novel variants in mice and potentially non-human primates.

97. Quantitative Analysis of Subretinal AAV-GFP in Fluorescent Fundoscopy Images from Non-Human Primates

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Purpose: Design and optimization of therapeutic vector constructs, including capsid serotypes and promotors, typically evaluates vector efficacy via reporter genes such as GFP and mCherry. Ocular vector constructs are frequently gauged based on fluorescence fundus images (in-life) and immunohistochemistry (termination). Evaluation of reporter gene expression at different timepoints and in different eyes using fluorescence fundus images requires reproducible image capture settings. Variables known to affect fluorescence intensity in images include instrument, exposure, gain, angle, focal plane, bleb geometry, eye geometry, level of mydriasis, intrinsic autofluorescence, and pattern of fluorescent reporter gene expression. Many of these variables cannot be controllably reproduced. We developed a quantitative method that normalized the signal to background (fields lacking reporter gene expression) within each image. This enabled a quantitative comparison among different vector constructs. **Methods:** Three common serotypes of AAV that are used in ocular clinical trials were manufactured with a GFP reporter gene using triple transfection technology. Twelve Cynomolgus monkeys (Macaca fascicularis) underwent bilateral subretinal injection of 4.5x10e10 vg/eye in 100 µL. Fundus fluorescence images were collected at pre-dose and 2, 4, 6, 10, and 13 weeks post-dose using a Heidelberg cSLO SPECTRALIS® instrument. Settings for each image were adjusted to avoid overexposure with focus on constant features such as retinal blood vessels. A minimum of 2 images were collected per eye, one of the posterior pole (includes the optic nerve and fovea) and one of the bleb. Additional images to capture the borders of the bleb and retinal tissue outside of the bleb area were collected. Quantitation was performed using ImageJ software. A circular polygon region was used to sample the average GFP fluorescence intensity of regions outside (background) and within the bleb. Per image, the average background fluorescence was subtracted from the average bleb intensity, and then a ratio of signal to background was calculated. **Results:** At 2 weeks after injection, the GFP fluorescence signal remained close to background level for all three AAV capsid serotypes. At 4 weeks after injection all three serotypes had GFP signals above background, and an apparent difference in the rate of increase of expression began to emerge. Peak increase in GFP signal occurred between 4 and 6 weeks and was followed by a leveling off after 10 weeks. **Conclusions:** We developed a quantitative method for comparative analysis of subretinal AAV-GFP in fluorescence fundus images that revealed apparent differences in onset and rate of expression among three capsid serotypes. Differences were detected between images that appeared similar to an observer performing a qualitative assessment. Although this method was developed using GFP expression in non-human primate eyes after subretinal injections, the same method could be extended for use with other fluorescent labels, images from other species or injection through other routes. We are exploring similar methods to analyze GFP expression following intravitreal dosing.

98. Analysis of Expression and Activity of Wild-Type and Mutant RPE65 Proteins *In Vitro* and *In Vivo*

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The enzyme retinal pigment epithelial 65 (RPE65) converts all-trans-retinyl ester to 11-cis-retinol in retinal pigment epithelial (RPE) cells and is critical for the visual cycle. Mutations in the *RPE65* gene are associated with inherited retinal dystrophy with a number of clinical descriptors including Leber's congenial amaurosis (LCA) and autosomal recessive retinitis pigmentosa (RP). We have developed LUXTURNA (voretigene neparvovec-rzyl), the first gene therapy for the treatment of patients with confirmed biallelic *RPE65* mutation-associated retinal dystrophy, and the *first adeno-associated virus (AAV) vector gene therapy approved in the U.S.* The current study aimed to assess the expression of a high specific activity RPE65 mutant protein. It has been shown that chicken RPE65 has higher isomerohydrolase activity than the human enzyme *in vitro* (Moiseyev G, JBC 2008). If five amino acids of the wild-type human RPE65 protein are substituted with the corresponding ones from the chicken RPE65, the human RPE65 mutant gains activity comparable to the chicken enzyme. To study this finding in more detail, we generated AAV2-wild-type-hRPE65 and AAV2-mutant-hRPE65 vectors and compared RPE65 activity *in vitro* in HEK293A LRAT cells, and *in vivo* in *rd12* mice (mice with null mutation in *RPE65* gene). HEK293A cells were transduced with AAV2-wild-type-hRPE65 or AAV2-mutant-hRPE65 vectors at a multiplicity of infection (MOI) of $1x10^5$ vg/cell. While mRNA levels of wild-type and mutant *RPE65* were similar, the mutant protein was 2-fold higher than the wild-type. This was associated with a 2-fold increase of isomerohydrolase activity. To test the effect on retinal function and to compare the activity of wild-type and mutant RPE65 *in vivo*, *rd12* mice received bilateral, subretinal injections with $1x10^9$ or $1x10^{10}$ vg/eye of either AAV2-wild-type-hRPE65 or AAV2-mutant-hRPE65. At baseline, there were no differences in electroretinogram (ERG) amplitudes across the *rd12* treatment groups prior to subretinal

administration. One month post injection, each of the four groups of mice receiving subretinal administration of AAV2 vectors had significantly higher scotopic wave amplitudes relative to the mice receiving vehicle. There was a significant rescue of photopic b-wave amplitudes in the treatment groups receiving $1x10^9$ vg/eye of either AAV2-wild-type-hRPE65 or AAV2-mutant-hRPE65. There was no difference in ERG amplitudes between groups injected with wild-type or mutant vectors. RPE65 mRNA and protein levels increased in a dose-dependent manner, but there was no significant difference between wild-type and mutant RPE65. Finally, isomerohydrolase activity was detected in *rd12* mice injected with either AAV2-wild-type-hRPE65 or AAV2-mutant-hRPE65 with dose-dependent increases, but there was no difference between the two vectors. Overall, our data demonstrate that despite the higher activity of the mutant RPE65 observed *in vitro*, this effect did not translate *in vivo*.

99. Transduction Efficiency of AAV Vector is Increased in Cell Lines with Overexpressed AAVR

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AAVR is a key receptor for AAV vector transduction. We hypothesized that overexpression of AAVR in cell lines could increase the transduction efficiency for AAV vectors. Such more permissive cell lines could be useful to increase the sensitivity of AAV-related assays such as neutralization antibody titration. For this purpose, AAVR was overexpressed in HeLa cells by a lentiviral vector. The stable cell lines (termed as HeLa-OA) were selected using puromycin and AAVR overexpression was confirmed by Western blotting. Different serotype1-10 of AAV vectors carrying eGFP reporter under the control of CB promoter were tested to transduce HeLa-OA. Flow cytometric analysis showed that the transduction efficiency increased 2-10 folds for all serotypes except AAV4 which could not infect HeLa cells. We then used HeLa-OA to check the neutralization antibodies in non-human primate and human plasma samples for AAV1, 2, 5, 8 and 9 which have important clinical applications. The results showed that the sensitivity of neutralization antibody titration was greatly improved by the use of HeLa-OA. All together, it was suggested that construction of the cell lines with overexpressed AAVR could be a useful approach to improve the related assays for AAV vectors.

100. Biophysical and In Vitro Comparability Analysis of an AAV Vector Produced by the Baculovirus/Sf9 System and HEK Triple Transfection System

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Many products that are currently in phase I/II clinical trials have been manufactured using platforms such as the HEK293/Triple Transfection system. This platform is a reliable manufacturing system for rAAV production, but a more scalable process is required to support late stage clinical and commercial demands. The baculovirus/Sf9 production system for rAAV has been shown to be a scalable system that can produce large quantities of high quality rAAV product. To bridge the materials between these two manufacturing systems, comparability must be established. We have compared two AAV2.AADC vectors produced using the Bac/Sf9 and HEK/TT system head-to-head using a suite of biochemical, biophysical, and bioassay methods. Some methods that were used to compare the vectors include vector potency, infectivity, identity, residual protein and DNA analysis, and empty/full analysis. The rAAV vectors produced in the HEK/TT and the Bac/Sf9 systems were found to have comparable quality across multiple parameters. When comparing the efficacy of the vectors they were determined to have indistinguishable potency in vitro and in vivo. The materials produced using the two manufacturing systems were determined to be comparable.

101. Oversized Recombinant Adeno-Associated Virus (rAAV) Vectors Are Associated with Decreased Capsid Stability

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Recombinant adeno-associated viral (rAAV) vectors have had considerable success as delivery tools for the therapeutic treatment of genetic diseases. One of the major components of an AAV vector is the capsid, which plays a key role in this delivery process, and dictates tissue tropism, immunogenicity, and transduction efficacy. In the present study, the differential thermostability associated with rAAV vectors in relation to their nucleic acid content was examined. To begin, the response of AAV vectors containing different genome sizes to heat treatment was tested, and the amount of AAV genomes released upon thermal treatment was quantitatively measured. The results of a Quantitative real-time PCR (qRT-PCR) assay revealed that only a minority of genomes (10-16%) could be detected at 75°C-99°C for normal sized rAAV particles (genome size ≤4.7kb), and that the number of detectable genomes was time and/or serotype independent. In contrast, almost all the genomes (70~100%) for oversized rAAV particles (genome size ≥5.0kb) were detected at 75°C-99°C. Furthermore, agarose gel electrophoresis demonstrated that the release of genomes from normal sized rAAV vectors was temperature dependent, with no genomes released at the low temperature of 55°C -65°C, and 20% of genomes released at approximately 75°C. The permeability of the normal sized rAAV particles capsids was enhanced, according to the results of an ethidium bromide (EB) assay. Interestingly, the total amount of AAV genomes released increased proportionally with vector DNA size and the temperature of heat treatment. These results demonstrated that the normal sized rAAV particle capsids can sequester its enclosed DNA, and protect it from enzymatic reactions, even after heat inactivation. Conversely, the oversized rAAV particles release their DNA readily. However, any protecting effects of the heat denatured rAAV capsid are nullified upon the addition of typical lysis buffer. These findings suggest that the spatial structure of normal sized AAV particle capsids are different from oversized AAV particle capsids. This has interesting implications

regarding the biophysical mechanisms underlying rAAV uncoating and genome release, and provides a foundation for future improvement on vector production, storage and usage.

102. Optimization of the Triple Transfection Process in Suspension Cells Using a Novel Cationic Agent for Potent AAV Production in Large-Scale Bioreactors

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Triple transfection of adherent HEK293 cells is the gold standard method to produce AAV vectors but delivers limited amounts of product. To improve scalability of this process, we previously adapted adherent HEK293 cells to grow in suspension culture in chemically defined culture medium. Using Poly Ethylene Imine (PEI) as transfection agent, we could set up an AAV manufacturing process at 200L scale. However, the productivity was around 1E13 vg/L which was still not sufficient to address vector demands for some genetic diseases like neuromuscular disorders. Consequently, we focused our efforts on two limiting parameters of the process: the cell line itself and the transfection efficiency of PEI. More than 400 single cell clones of HEK293T were isolated and expanded in suspension using serum-free medium. From 30 clones that were evaluated for transfection efficiency, 2 produced 10-fold higher titers of AAV. In parallel, a novel cationic polymer was evaluated for transfection. While the transfection efficiency was not significantly higher than PEI in the high-producer clones, the vector titers were further increased. Strikingly, the ratio of full/empty capsids was dramatically improved since the optimized conditions generated AAV8 and AAV9 products containing more than 60% of full particles. The results were confirmed in stirred tank bioreactors. The process was scaled up to 50L single use bioreactor with an AAV9 vector leading to titers up to 1E14 vg/L with 60% of full capsids. This triple transfection process is reproducible and is currently further scaled up to 200L bioreactors.

103. Most Informative Characterization of Viral Vectors for Gene Therapies Can Be Achieved by Combining Different Analytical Methods

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Viral vectors have emerged as safe and effective delivery vehicles for clinical gene therapy. Manufacturing of clinical grade vectors requires knowledge of the complex methods needed to generate, purify and characterize viral vectors. One of the most crucial, but also problematic steps, is the characterization of viral vectors in different production phases during process development and in final drug substance. Molecular methods such as quantitative real-time PCR (qPCR) and digital PCR (dPCR) enable relative and absolute quantification, respectively, of target DNA or RNA. qPCR is, due to its capability to detect nucleic acids in traces, widely used for detection and relative quantification of various targets, from viral nucleic acids, to residual DNA. On the other hand dPCR offers many advantages that might provide more accurate quantitative result. Our experiments have shown that dPCR is less sensitive to impurities originating from sample matrix. Additionally, we have shown that dPCR is accurate, robust and repeatable in qualified or validated assays (coefficient of variation usually below 10 %). Nevertheless, the presence of viral or construct genome doesn't mean that the virus particles are actually full and intact. To complement results of molecular methods, we use transmission electron microscopy (TEM) for direct observation and counting of virus particles. With TEM analysis we evaluated viral structure, presence of full and empty/damaged/broken capsids and presence of impurities in various intermediate samples. Finally, to characterize the presence of other nucleic acids in the sample than the desired ones, we introduced Next Generation Sequencing. We were able to identify the impurities present in the samples of viral vectors, which were not only the sequences targeted by host cell DNA or resistance gene qPCR assays, but also other sequences, indicating that current assays might be underestimating the residual DNA quantity. The performance of different methods for characterization of viral vectors will be presented in light of different sample matrices. Insights will be given on how the combinations of methods used can improve the overall sample characterization.

104. Characterization of a Dimeric Transgene Isoform and Its Potential Impact on AAV Product Quality

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Background: The undesirable outcome of Factor IX (FIX) gene therapy Phase 1/2 clinical trial with BAX 335 led to a development of second generation of FIX construct. The second generation was developed as a single stranded FIX vector with the same capsid, adeno-associated virus subtype 8 (AAV8). The manufacturing process for new vector was optimized and the aim was to establish a scalable process that yields vector batches of consistently high purity and yield. A pre-requisite for achieving this goal is profound process and product understanding. Therefore, vector preparations generated during process development were characterized using a comprehensive panel of analytical methods. In the course of vector material characterization, it was also analyzed for transgene integrity. This was resulting in the identification of an AAV sub-population containing a dimeric transgene isoform that was subjected to further characterization. Aim: The goal of the study was to investigate the vector sub-population with encapsidated dimeric DNA isoform, checking for its biological activity and evaluating if the degree of this sub-population can be successfully reduced in the final product in order to increase overall product homogeneity. Methods: The vector material was produced by HEK293 cell line with triple transient transfection. The production process employed downstream purification with an ultracentrifugation step. The dimeric isoform of the transgene was detected in a defined high density zone of the

ultracentrifugation process. This fraction was enriched and purified in order to obtain a vector population that primarily contains dimeric DNA for further analysis (qPCR, ddPCR, analytical ultracentrifugation, native and alkaline agarose gel electrophoresis, restriction digest, size exclusion chromatography, *in vitro* potency (FIX), *in vivo* potency FIX gene therapy vector in FIX ko mice). Results: Analytical ultracentrifugation of vector preparations enriched with AAV containing dimeric transgene isoform showed following species: full AAV capsids containing monomeric transgene at 70S (sedimentation coefficient), full AAV subpopulation containing dimeric transgene isoform at 81S - 85S, and oligomeric AAV capsids detected at 95-105S. Measurement of the *in vitro* and *in vivo* potency demonstrated that the material is biologically active. However, comparison of the potency of AAV populations containing monomeric or dimeric transgene DNA has not been fully resolved yet. Consequently, dimeric transgene species may pose risk to overall product quality. In order to increase product homogeneity this AAV sub-population was omitted for manufacturing scale production runs. Initial AUC results indicate that this consistently reduces the percentage of AAV containing dimeric transgene to a level below 3% of the total peak area. Conclusion: Our proprietary large scale ultracentrifugation process is capable of reducing AAV subpopulation containing dimeric DNA isoform which may pose risk to the overall product quality to an acceptable level.

105. Automated Determination of the Purity and Packaging of Gene Therapy Delivery Platforms Using Transmission Electron Microscopy

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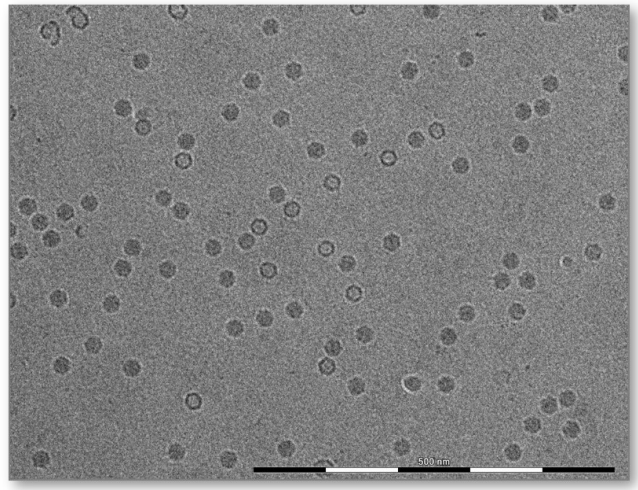
The morphological characterization of gene therapy delivery platforms can be used as a support throughout the whole drug development process, by identifying typical product-related impurities and analyzing of the capsids packaging supports. Indeed, gene delivery empty viral capsids may be the result of failure in packaging or occur as a result of structural changes of the capsid along the manufacturing process - the latter being not only a risk for lowered potency but also representing a safety issue for the patient, as particles with a damaged capsid can provoke immunogenicity issues. Transmission electron microscopy (TEM) offers a wide range of analysis methods that can provide unique insights in the characterization of viral gene delivery platforms such as adeno-associated viruses (AAVs), adenoviruses and retroviruses. The choice of method depends on whether potency (ratio of viral capsids containing a gene) or purity (absence of non-intact particles) is assessed. TEM analysis of negatively stained samples (nsTEM) can provide insights on the morphology of the viral vectors present in a specimen, where a qualitative assessment can be made on the intactness of the particles, and on the presence and characterization of impurities. MiniTEM, a compact low voltage electron microscope with automation features specifically designed for the characterization of nanoparticles can be used to automatically analyze specimen and give indications on the integrity of the particles and on the presence and nature of process related impurities. TEM analysis of Cryo-preserved samples (cryoTEM) is used to assess the encapsulation of vectors such as AAV and is, in combination with the Vironova image analysis software (VAS), a GMP validated method suitable for QC testing of the ratio of full AAV particles. In this presentation, specimen characterization and results obtained from automated analyses using both these techniques will be discussed, and several examples on how automated TEM imaging and image analysis can provide robust and reliable data for the research, development, and production of gene delivery platforms will be presented.



106. Comparison of Viral Production, Biodistribution in Mice and Gene Delivery in Human IPS-Derived Myotubes of Different AAV Serotypes

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Recombinant Adeno-Associated Virus Vectors (rAAV) are widely used tools for gene delivery. The different AAV serotypes can transduce a wide range of biological tissues and organs but with variable tropism. The yields for the production of different rAAV serotypes are also variable. A challenge is therefore to identify the most pertinent serotype for the intended application. To identify the best rAAV for the gene therapy of muscular dystrophies, we compared rAAV using different serotypes (AAV8, AAV9 and rh74) measuring their productivity, biodistribution in mice and capability to transduce cultured human iPS-derived myotubes. According to our bioproduction processes, the AAV9 serotype was the most efficiently produced. rAAV expressing luciferase were injected in the tail vein of wild-type albinos mice at two different doses. Bioluminescent images of whole mice were collected after 15 and 30 days and the major organs were sampled at the end-point of the experiment for quantification of luciferase on protein extracts and of AAV DNA by quantitative PCR. Whereas AAV transduction appeared to be almost equivalent in the different organs regardless of serotypes, AAV9 seemed to induce a higher luciferase expression in most of the transduced organs except in the liver. In human cells *in vitro*, the highest levels of transduction of myotubes was obtained

with rAAV8 and 9. These results provide a baseline set of results to further define the optimal rAAV and will also serve to select new capsid mutants with improved specificity for muscular dystrophies.

107. Engineered Triple Mutant AAV6 Capsid, AAV6.2ff, Mediates Rapid and Robust Transgene Expression in the Muscle and Lungs of Mice

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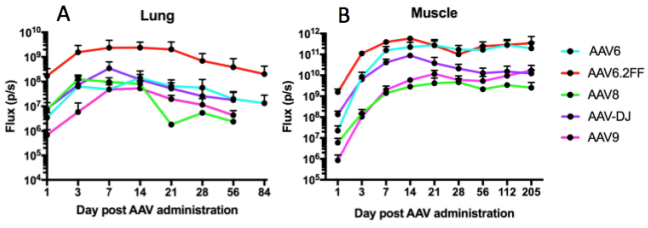
AAV6 is a commonly selected AAV serotype for targeting the muscle and the lungs. AAV6.2FF is a triple mutant AAV6 capsid comprising F129L, T445F and T731F point mutations. The AAV6.2FF capsid was engineered with the aim of generating an improved vector for transducing both muscle and lung tissue for gene therapy and vectored immunoprophylaxis platforms. AAV6.2FF maintains the heparin sulfate binding profile that is associated with AAV6 while also improving *in vitro* cell line transduction 7-39 fold over the parental AAV6. Anecdotally, increased production yields were observed for AAV6.2FF pseudotyped vectors and quantification experiments are ongoing. AAV6.2FF-mediated expression of alkaline phosphatase in the mouse lung following intranasal administration was 10X increased compared to AAV6. Co-localization follow up experiments revealed AAV6.2FF transduces alveolar type II cells (pro-SPC+) in addition to CD45+ cells. A separate experiment using an *in vivo* imaging system (IVIS) comparing AAV6.2FF, AAV6, AAV8, AAV9 and AAV-DJ pseudotyped luciferase vectors demonstrated AAV6.2FF mediated the strongest expression in the lung over 84 days (fig 1A). A similar experiment was conducted using the same five luciferase vectors administered intramuscularly. AAV6.2FF mediated the most rapid signal reaching 20% of the signal maximum by day 3, while AAV6 only reached 4% of its maximum at the same time point. However from 21-205 days post injection, AAV6 and AAV6.2FF-mediated luciferase expression in the muscle were comparably high in contrast to AAV8, AAV9 and AAV-DJ (fig 1B). Interestingly, AAV8 and AAV9 mediated extramuscular luciferase expression in the abdominal region of the mice, which was not observed for AAV6.2FF, AAV6 or AAV-DJ. Ongoing experiments aim to elucidate the mechanism for this vector migration.



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108. Novel Phosphorylation Sites in the CD28 Costimulatory Domain Shape CAR-T Cell Function

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Chimeric antigen receptors (CARs) contain an antigen-sensing ectodomain and a signaling endodomain. The endodomain is responsible for the initiation of a phosphorylation cascade that results in activation of the T-cell proliferative and cytolytic programs. Numerous attempts to optimize the signaling properties of CAR endodomains have been described, mostly focusing on the expected downstream signaling events. However, a global, system-level assessment of the CAR-triggered signaling network has not been described. In order to conduct an unbiased analysis of CAR-initiated signaling events, we designed a phosphoproteomic assay in which PSCA-specific CAR-T cells (CAR-Ts) are stimulated with metabolically heavy labeled pancreatic cancer cells that naturally express PSCA. Phosphorylation events (pY and pS/T) were detected by LC-MS/MS in the co-culture extracts. Post-hoc analyses allowed us to discriminate the signal corresponding to T cells, based on exclusion using the shift of mass/charge ratio observed for heavy isotope-labeled tumor proteins. We found 40 peptides (of 791) that were differentially phosphorylated between CAR-T and mock-transduced T cells, spanning multiple signaling pathways. Following recognition of tumor cells, 2nd generation CAR-Ts exhibited more pronounced changes in phosphorylation than 3rd generation counterparts. Interestingly, we detected higher phosphorylation in all four tyrosine (Y) residues contained in the CD28 domain. Two of these Y residues (Y191/YNMN and Y209/PYAP) are well characterized in terms of their functional relevance. In contrast, the role of residues Y206 and Y218 is poorly understood. In order to evaluate their relevance in the setting of CAR signaling, we generated 4 different versions of the anti-PSCA CARs, each of which included an alanine-substitution in one of the identified Y residues. Upon transduction of human primary T cells, mutant CARs were expressed at similar levels, except for the Y218A mutant, whose expression was markedly inferior. CAR-Ts with mutation of Y191 exhibited similar production of IFNγ as the wild-type (WT). However, CARs harboring mutations in Y206, Y209 and Y218 exhibited a significant reduction of IFNγ release upon co-culture with tumor cells. Moreover, mutations in Y218 and Y206 severely impaired the ability of CAR-Ts to produce IL-2 in response to antigenic stimulation, while mutation in Y191 resulted in higher production of IL-2 compared with WT CAR-Ts. No significant differences were observed for CAR-Ts carrying mutation in Y209. In addition, although all the CAR-Ts carrying a CD28 with non-phosphorylatable substitutions exhibited a reduced proliferation rate in response to PSCA-expressing tumor cells, the Y218A mutant showed the lowest proliferation after tumor co-culture. Most importantly, human WT CAR-Ts and CAR-Ts carrying the Y191A mutation were equally able to control tumor growth in an *in vivo* model of pancreatic

adenocarcinoma, while CAR-Ts carrying mutations in Y206 and Y209 only partially control and Y218 failed to control tumor growth. In summary, we developed a mass spectrometry platform for the assessment of global signaling events in CAR-Ts in co-culture with tumor cells. Moreover, we more deeply characterized Y residues present in the CD28 co-stimulatory modules, and demonstrated that they play a key role in CAR expression and/or function. A deeper understanding of the molecular events controlled by these phosphosites in CAR-Ts will allow us to design new CARs with enhanced, and perhaps more tightly controlled, functional properties. We anticipate that this strategy will facilitate the study and rational design of next generation CAR-T cell products.

109. Enhancing Car T Cell Activity by Linking Il-12 Expression to the Endogenous Pdcd1 Promoter

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Chimeric antigen receptor (CAR) T cells have shown great promise in treating certain late stage hematological malignancies. While very encouraging, current CAR T cell therapies have not shown the same level of success in targeting solid tumors and alternative approaches are required to achieve clinical efficacy in solid tumor patients. We describe a combinatorial approach whereby targeted gene deletion and transgene insertion occur simultaneously resulting in more potent CAR T cells for solid tumor applications. Immunomodulatory cytokines can stimulate vigorous antitumor responses and are candidates for increasing CAR T cell efficacy in solid tumors. However, the clinical application of cytokine therapy has been limited by systemic toxicity, particularly for strong effector cytokines such as IL-12. Limiting IL-12 expression to within the tumor microenvironment may reduce unwanted toxicity while enhancing CAR T cell functionality. Immune checkpoint gene programed cell death 1 (*PDCD1*) is a regulator of T cell functionality that is highly upregulated following T cell activation, with antibody and nuclease-mediated inactivation of the PD-1 signaling pathway having been shown to enhance CAR T cell functionality. Here, we used megaTAL genome editing/homology directed repair (HDR) to place an *IL-12* transgene under the control of the *PDCD1* promoter, linking IL-12 production with CAR T cell activation as well as eliminating PD-1 expression. CAR expression was combined with site specific transgene expression as follows: lentiviral vector-engineered T cells were treated with a *PDCD1*-specific megaTAL and transduced with adeno associated virus-6 (AAV6) containing a promoter-less *IL-12* transgene flanked by *PDCD1* homology regions. We observed highly efficient HDR, with inducible IL-12 expression from the endogenous *PDCD1* promoter being dependent on T cell activation. Minimal IL-12 production was detected under resting conditions, whereas PMA/Ionomycin or co-culture with CAR+ target cell lines resulted in higher IL-12 secretion. Expression of IL-12 under the *PDCD1* promoter enhanced CAR T cell cytokine production and cytotoxicity, especially under conditions of repeated antigen exposure. In summary, we describe a novel genome editing strategy to enhance CAR T cell functionality. Using HDR, we were able to engineer CAR T cells to simultaneously disrupt the *PDCD1* gene and place a potentially therapeutic transgene under inducible transcriptional control. The IL-12/CAR T cells exhibited activation-dependent IL-12 production and enhanced cytokine and cytotoxicity responses against tumor cells *in vitro*. HDR may represent a promising approach to enhance CAR T cell functionality in solid tumor applications.

110. Modulating IL-7 Cytokine Receptor Signaling to Enhance the Persistence and Anti-Tumor Efficacy of Epstein-Barr Virus (EBV) Specific T-Cells in EBV Positive Malignancies

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Up to ~40 % of Hodgkin and non-Hodgkin lymphomas (HL and NHL) carry the Epstein-Barr virus (EBV) genome in a latent state involving the expression of latent viral proteins EBNA-1, LMP-1, LMP-2 and BARF-1 (type 2 latency antigens [T2Ags]). T-cells specific for T2Ags (T2EBVSTs) can be generated from the blood of EBV positive individuals including HL and NHL patients. Our lab has used T2EBVSTs to target EBV positive malignancies with promising results. However, lack of cytokines to support T-cell growth in vivo, together with a suppressive tumor microenvironment (TME) that includes suppressive myeloid and regulatory cell types, are major challenges to the persistence and efficacy of tumor-specific T-cells. Interlukin-7 (IL-7) has both growth promoting and anti-apoptotic effects on T-cells and may be exploited for the benefit of EBVSTs. However, the IL-7 receptor is downregulated on activated T-cells and IL-7 has limited availability in vivo. Our lab has recently demonstrated that transgenic expression of a constitutively active IL-7 receptor (C7R) induces STAT5 signaling and enhances the expansion, persistence and anti-tumor efficacy of T cells modified with chimeric antigen receptors (CARs) for neuroblastoma and glioblastoma in xenograft models. Hence, we hypothesize that EBVSTs expressing C7R to enable cytokine-independent proliferation and resistance to the TME will have increased potency, persistence and efficacy in EBV+ malignancies. I have generated T2EBVSTs expressing C7R (55% ± 20% transduced CD3+ EBVSTs, n=3), with stable specificity for T2EBV antigens as demonstrated by enzyme-linked immunospot (ELISpot) assay (4046 ± 2438 vs. 4114 ± 2374 spots per 10^5 T2EBVSTs; C7R-T2EBVSTs vs. unmodified T2EBVSTs). To evaluate the effects of C7R on the anti-tumor effects of T2EBVSTs, I compared the anti-tumor efficacy and persistence of C7R-EBVSTs vs. EBVSTs in human cell line derived xenograft mice model. I established subcutaneous EBV transformed human B-cells (EBV-LCLs) in NSG mice and 8 days later (average tumor volume of 130 mm^3), infused C7R-EBVSTs or EBVSTs or no T cells. Mice receiving C7R-T2EBVSTs exhibited tumor clearance by day 60, while the tumor volume of mice receiving T2EBVSTs without C7R was 240 ± 62 mm^3; $p<0.000$, n=5/group). Control mice that did not receive EBVSTs were sacrificed on day 39 (Tumor volume 602 ± 67 mm^3). C7R-T2EBVSTs also demonstrated enhanced persistence (67 days compared to 27 days for unmodified EBVSTs). The demonstration of increased persistence and enhanced anti-tumor efficacy of T2EBVSTs expressing C7R in our EBV positive tumor model provides a novel and

innovative platform for translating our findings to future clinical trials. The effects of IL-7 signaling in resisting immunosuppressive networks suggest that IL-7 receptor induced STAT5 signaling may abrogate some effects of suppressive TME. In future experiments, we will elucidate the mechanisms underlying the protective effects of C7R in the presence of individual cellular and molecular components of the suppressive TME such as MDSCs, M2 macrophages and T-regs and certain molecular components, such as amino acid depletion, hypoxia and oxidative stress.

111. PD-L1 Checkpoint Blockade Using a Single-Chain Variable Fragment Targeting PD-L1 Delivered by Retroviral Replicating Vector Enhances Anti-Tumor Effect in Cancer Models

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A proprietary retroviral replicating vector (RRV) platform based on an amphotropic gamma retrovirus that preferentially infects and replicates in tumor cells has been developed to address historical challenges with viralbased treatments for cancer. Our lead program, Toca 511 (vocimagene amiretrorepvec, an RRV encoding cytosine deaminase) used in combination with an oral prodrug, Toca FC (extended-release 5fluorocytosine), recently entered a Phase 3 trial in patients with recurrent high grade glioma (NCT02414165). In Phase 1 trials involving 127 patients, this product demonstrated durable, complete responses and a favorable safety profile. In preclinical studies, PD-L1 checkpoint blockade is explored using the tumor-selective gene delivery system. Immune checkpoint inhibitors are a breakthrough for immunotherapies in treating cancer patients. However, the response rate ranges from 20-30% across tumor types with a number of immune-related adverse events associated with treatment which can lead to a high rate of treatment discontinuation (~ 12-39% patients). An RRV expressing a singlechain variable fragment targeting PDL1 (RRVscFvPDL1) has demonstrated that scFvPDL1 binds specifically to both mouse and human PD-L1, and the binding specificity of scFvPDL1 was further confirmed by competitive ELISA showing that RRVgenerated scFvPDL1 was able to compete for target occupancy against a commercially available monoclonal antibody against PDL1. A bystander effect also has been observed with scFvPDL1 protein expression from RRVscFvPDL1 infected tumor cells in a dose-dependent manner showing saturated receptor binding to the cell surface PDL1 of bystander cells when co-cultured with as low as 10% scFvPDL1 expressing cells. In addition, *in vivo* mouse models showed that tumor cells infected with RRV-scFv-PDL1 conferred robust and durable immune-mediated antitumor activity superior to systemically administered anti-PD1 or antiPDL1 monoclonal antibodies. These results support that RRVscFvPDL1 checkpoint inhibition is potentially therapeutic and may provide an improved safety and efficacy profile compared to systemic monoclonal antibodies. The anti-tumor activity of RRVscFvPDL1 may be a consequence of the delivery approach, which provides a consistent high level of payload and bystander index which is localized within the tumor microenvironment. Furthermore, with selective local production, RRVscFvPDL1 may be therapeutically beneficial in combination with other entities as an immuno-oncology agent with less concern of combined autoimmunity adverse events.

112. A Reporter Cell Platform for High-Throughput Screening of Chimeric Antigen Receptor (CAR) Libraries

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Background: Chimeric antigen receptor-modified T-cells (CAR-T) are under intense investigation in cancer immunotherapy. Several parameters in CAR design including binding domain affinity and extracellular spacer length affect CAR-T function. At present, the approach to identify CAR constructs with optimal function is empiric testing in primary T-cells, which is time consuming and error prone due to variations in receptor expression and subset composition in T-cell lines. Here, we established a CAR screening platform that involves a stable reporter cell line, and demonstrate the ability to rapidly identify CAR variants with unique properties from large CAR libraries in high-throughput fashion. **Methods:** Reporter cells were derived from the immortalized Jurkat T-cell line and modified with NF-κB and NFAT-inducible CFP and GFP reporter genes. CARs were stably integrated in reporter cells, which were then activated with stimulator cells expressing the target antigen to analyze reporter gene-activation by flow cytometry. **Results:** To validate our reporter cells, we transduced them with a prototypic ROR1-specific CAR (R12 epitope, short IgG4-Fc spacer, 4-1BB co-stimulation) and performed stimulation with $ROR1^+$ target cells. We detected high levels of CFP and GFP reporter signal after 24 hours and a peak signal at 48 hours. Western blot analyses showed that ROR1-CAR stimulation activated NF-κB and NFAT in primary T-cells as well, thus confirming the importance of both transcription factors in CAR induction. Then, we screened a mini-library of n=3 ROR1-CARs that differed in spacer length (IgG4-Fc long-medium-short), based on our previous empiric analysis in primary T cells showing that the long spacer design was optimal for recognition of the R11 ROR1 epitope. The reporter cells readily identified the optimal CAR construct with long spacer based on CFP and GFP reporter gene expression, and reduced the analysis time from ~3 weeks (with primary T-cells) to <6 days. Next, we challenged our reporter platform to a CAR library screen with ~$2x10^5$ receptor variants. This library was derived from the R11 ROR1-CAR through random PCR that introduced amino acid changes to the CDR3 region of the VH chain in the CAR binding domain. This approach is anticipated to generate few variants with higher or lower affinity compared to wild-type, and a high number of drop-out variants that lose specificity for ROR1. About $6x10^7$ reporter cells were transfected with this library and positive reporter cells were enriched from 6% to 47% using an EGFRt selection marker. We activated these cells with $ROR1^+$ stimulator cells and performed single cell sorting of CFP^+ GFP^+ reporter cells, which established 20

clones that generated a strong reporter signal in response to ROR1 antigen. CDR3 sequencing revealed 3 unique CAR variants that are currently functionally validated in primary human T-cells and analyzed for affinity in comparison to the wild-type ROR1-CAR. **Conclusions:** We established a versatile CAR screening platform with NF-κB and NFAT reporter cells. Our data show that this platform can be utilized to identify CAR constructs with unique properties from large CAR libraries that are otherwise not amenable to evaluation in primary human T-cells. We are currently adapting this platform for screening campaigns on naive antibody libraries to identify CAR constructs that recognize novel tumor antigens.

113. Exogenous In Vitro and In Vivo Regulation of Interleukin-12 Secretion from T Cells Using Destabilizing Domain Technology

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Chimeric antigen receptor (CAR)-engineered T cells (CAR-Ts) have generated remarkable efficacy in eradicating B cell malignancies such as B cell acute lymphoblastic leukemia (B-ALL). However, CART-T therapies have yet to be successfully applied to solid tumors, due to the hurdles of on-target/off-tumor recognition of normal tissues, low CAR-T cell expansion and persistence, the immunosuppressive tumor microenvironment, and clonal heterogeneity within tumors. Enhancing CAR-T cell functionality, and selectively delivering cargo to the site of solid tumors represent key tactics to achieve effective CAR-T therapy for solid tumors. In particular, Interleukin-12 (IL-12) has been of great interest given its potential to remodel the tumor microenvironment. Indeed, IL-12 has previously been shown to enable efficacy of CAR- or T cell receptor (TCR)- modified T cells in preclinical tumor models, as well as tumor infiltrating lymphocytes (TILs) in clinical trials. However, constitutive production of IL-12 can compromise safety and/or efficacy, such that on-demand, local delivery of the cytokine could be beneficial. To develop exogenously regulated IL-12, we utilized previously described destabilizing domain (DD) technology to regulate proteins *in vitro* and *in vivo* using small molecules. Destabilizing domains (DDs) are mutant protein domains that are inherently unstable, but can be reversibly stabilized by ligand binding. Moreover, the conditional stabilization can be conferred to fusion partners of DDs, and can therefore be used to control transgene expression. To create an exogenously regulated IL-12 system, we used an FKBP-derived DD to create an IL-12-FKBP fusion protein. The fusion protein was secreted at very low levels from cell lines and primary human T cells, but could be robustly induced with FKBP-ligands, Shield-1 or Aquashield, in a dose- and time-dependent manner. We also confirmed the functional activity of IL-12-FKBP using both reporter cells and primary human T cells. We then transferred T cells that were lentivirally-transduced with IL-12-FKBP into NSG mice, and orally dosed the animals with vehicle or Aquashield. Plasma IL-12 levels in vehicle-dosed animals were barely detectable. In contrast, plasma IL-12 levels rapidly increased over 4-6 hours upon single-dose administration of Aquashield and then receded to baseline at 24hrs. Sequential Aquashield-induced pulses of IL-12-FKBP expression by the IL-12-FKBP transduced T cells also increased granzyme B and perforin expression by CD8+ T cells *in vivo*. These data demonstrate the potential of DD-dependent small molecule-driven exogenous control to safely enable the use of IL-12 in adoptive cell therapy.

114. CRISPR/Cas9-Mediated Knockout of DGK Improves Anti-Tumor Activities of Human T Cells

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The efficacy of T cell therapy is inhibited by various tumor-associated immunosuppressive ligands and soluble factors. Such inhibitory signals turn specific T cell signaling pathways on or off, impeding the anti-cancer functions of T cells. Many studies have focused on PD-1 or CTLA-4 blockade to invigorate T cell functions through CD28/B7 signaling, but obtaining robust clinical outcomes remains challenging. In this study, we sought to potentiate T cell functions through increasing CD3 signaling by knockout of diacylglycerol kinase (DGK) using CRISPR/Cas9. DGK is an enzyme that metabolizes diacylglycerol to phosphatidic acid (PA). We found that knockout of DGK augments the effector functions of CAR-T cells via increasing TCR signaling in vitro. DGK knockout CAR-T cells were resistant to soluble immunosuppressive factors, such as TGF-β and prostaglandin E2, and sustained effector functions under conditions of repetitive antigen challenge. Moreover, DGK knockout CAR-T cells caused complete regression of U87MGvIII glioblastoma tumors in a xenograft mouse model. Collectively, our study shows that knockout of DGK effectively enhances the effector functions of CAR-T cells, suggesting that CRISPR/Cas9-mediated knockout of DGK could be applicable as part of a multifaceted clinical strategy to treat solid cancers.

115. The Antitumor Effectiveness of γδ T Cells is Enhanced When Combined with Stress-Inducing Chemotherapy

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γδ T cells are an attractive candidate for antineoplastic cell-based therapies because of their cytotoxic activity mediated through the cell surface receptors NKG2D and CD16. NKG2D facilitates the innate recognition of stress-induced ligands such as MICA/B and UL-16 binding proteins (ULBP). CD16 activates mechanisms of antibody-dependent cellular cytotoxicity (ADCC). We have developed a safe and efficient method for serum free expansion, storage, and genetic engineering of γδ T cells, and we have focused on the use of these *ex vivo* expanded cells to treat neuroblastoma (NB) and glioblastoma (GBM). Conventional therapy for high-risk relapsed neuroblastoma and high-grade gliomas includes the alkylating agent temozolomide (TMZ). We have shown that NB and GBM cell lines upregulate stress inducible ligands within hours following exposure to TMZ. As such, we have developed a combined chemotherapy and γδ T cell-based immunotherapy to exploit the upregulation of stress antigens and

tumor-targeted antibodies to facilitate tumor cell killing. We show that our γδ T cell product in combination with dinutuximab, a monoclonal antibody that binds GD2 on the surface of NB cells, facilitates a 30% increase in tumor cell lysis compared to γδ T cells alone. To test the *in vivo* effectiveness of *ex vivo* serum free-expanded γδ T cells, IMR5 NB cells were established in NSG mice, which were then treated with a regimen of 400 μg dinutuximab every 10 days (intravenous [IV]), 40 mg/kg of TMZ (intraperitoneal [IP]) every third day, and $2.5x10^6$ γδ T cells (IV) 8 hours after TMZ injection. No difference in tumor burden was observed when comparing untreated mice to mice treated with γδ T cells ± dinutuximab, indicating γδ T cells did not aid in tumor regression. In contrast, significant reduction in tumor volume was observed in mice treated with a combination of 40 mg/kg TMZ, plus *ex vivo* expanded γδ T cells and dinutuximab, showing the importance of chemotherapy-induced cell stress. We then tested a second TMZ-sensitive tumor model, GBM, but in this model we introduced an O6-methylguanine-DNA-methyltransferase (MGMT) transgene into the γδ T cells, thereby conferring resistance to TMZ. This drug resistant immunotherapy (DRI) platform allows for the combined administration of γδ T cell immunotherapy and TMZ chemotherapy. We established intracranial (IC) glioma xenografts using the human GBM xenografts X12 (Classical) and X22 (Mesenchymal) to investigate the benefit of DRI *in vivo*. After tumor growth was established, treatment consisted of 60 mg/kg TMZ (intraperitoneal [IP]) twice weekly and $1x10^6$ TMZ-resistant γδ T cells IC 4 hours after TMZ administration. Similar to our NB studies, there was no survival benefit in mice treated with γδ T cells alone. However, when TMZ was included, 80% long-term survival was achieved ($p = 0.0001$ vs TMZ alone). Interestingly, although not as robust, DRI plus TMZ treatment of established tumor in mice with a TMZ-resistant X12 cell line resulted in 10% of animals achieving long-term survival, compared to none in the cell only or TMZ only groups. Therefore, combining TMZ to induce stress ligands, in both NB and GBM models, significantly improves the cytotoxic effect of γδ T cells. These data show that chemotherapy in conjunction with cellular immunotherapy can improve the therapeutic outcome in preclinical cancer models and support future clinical testing.

116. Hunting WT1-Specific T Cell Receptors for TCR Gene Editing of Acute Myeloid Leukemia

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Gene transfer and genome editing technologies can render cancer immunotherapy feasible and effective in the treatment of several tumor types. By T cell receptor (TCR) gene editing, we showed that it is possible to completely redirect T cell specificity against a tumor antigen. Unfortunately, wide-range implementation of TCR-based immunotherapy in clinical practice is currently limited by the paucity of tumor-specific T cells and TCRs. Aim of this study is the generation of a library of TCRs encompassing different human leukocyte antigen (HLA) restrictions with specificity for acute myeloid leukemia (AML), an aggressive and still largely incurable haematological malignancy. Since most tumor-associated antigens are over-expressed self-antigens, high avidity tumor-specific T cells are rarely found *in vivo* as they are deleted or anergized. Still, spontaneous T cell responses against specific leukemia antigens have been observed in patients after allo-hematopoietic stem cell transplantation and in healthy donors. We focused on the identification of T cells specific for Wilms Tumor 1 (WT1), a tumor-associated antigen over-expressed in AML. We selected 7 healthy donors (HDs) to hunt for TCRs restricted for the 6 most frequent HLA class I alleles in the Caucasian population. Tumor-specific T cells were enriched by repetitively stimulating peripheral blood mononuclear cells (PBMCs) with autologous antigen-presenting cells (APCs: PBMCs or immortalized B cells) loaded with a peptide pool spanning the sequence of the tumor-associated protein. Expansion of tumor-specific T cells was determined by cytofluorimetric analysis of IFN-γ production and CD107a expression. The ability of WT1-specific T cells to recognize naturally processed epitopes and their on-target specificity was demonstrated upon co-culture with several antigen-expressing cell lines and primary leukemic blasts. The peptide specificity of isolated T cells was further identified by deconvoluting the responsive peptide pools. The expanded T cells were profiled by flow cytometry and TCR sequencing. Of note, by tracking overtime clonal contribution for each individual culture, without performing single cell sorting, we were able to identify and correctly pair 10 tumor-specific αβ TCR clonotypes recognizing tumor-associated peptides, restricted to different HLA alleles. Once expressed in T cells, the newly identified TCR reacted against antigen-expressing leukemic cells. Simultaneous CRISPR/Cas9-mediated knock-out of the endogenous TCR is ongoing to enhance expression and persistence of inserted WT1-specific TCRs. Overall, we developed a protocol consistently leading to the isolation of tumor-specific T cells from HDs. These optimized methodologies will streamline TCR hunting against other hematologic cancer antigens as well as targets in solid tumors.

117. Baboon Retrovirus Envelope Pseudotyped Lentivectors Permit Robust Transduction of NK Cells and Represent and Improved Tool for Cancer Immunotherapy

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NK cells, with their intrinsic ability to recognize and kill tumor cells represent an interesting tool for immunotherapy, even in an allogenic setting.. Although infusions of activated NK cells are promising for immunotherapy,there is room for improvement. The genetic modification of NK cells could strongly improve their functions. However, NKs are quite resistant to transduction with classical VSVG pseudotyped lentivectors (LVs). We therefore hypothesized that alternative lentiviral pseudotypes might result in more efficient transduction. Freshly isolated NK and NK obtained from the NK cell

Activation and Expansion System (NKAES) were transduced with a GFP-reporter gene using different lentiviral envelopes: baboon endogenous retrovirus (BaEV), RD114, MV-LV (lentiviral vector pseudotyped with the measles virus) and VSV-G (vesicular stomatitis virus). NK cells were then expanded using the NKAES system for 14-21 days and transduction was assessed by flow cytometry. BaEV, RD114 and MV receptor expressions were evaluated by RT-PCR and flow cytometry under different stimulation conditions. VSV-G-LVs resulted in poortransduction rate of freshly isolated NKs (0,28±0,12%) while RD114- and MV-LVs performed better (21±2,8% and 13±4,7%, respectively). The use of BaEV-LVs outperformed them all with a transduction rate mean of 30±2,2% in freshly isolated NKs and 87±6.7% in NKAES, even at low vector doses. Transgene expression was sustained stable for at least 21 days. BaEV transduction efficacy could be attributed to the BaEV receptors (ASCT1 /ASCT2) expression patterns As a proof of concept, we transduced chimeric antigen receptor (CAR) against CD22 or GD2. Sorted and re-expanded transduced NK cells kept their CAR expression, retained their cytotoxic functions against cancer cells. These BaEV-LVs represent a NK transduction technique that will allow the development of efficient NK-based immunotherapies such as CAR-NK cells or enhanced NK cells.

118. Targeting T-cell Leukemia with CD5-Based Chimeric Antigen Receptor-Modified γδ T Cells

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CAR T-cell therapy is an effective and approved treatment for relapsed/refractory B-ALL. The development of a memory T-cell response is one aspect of this therapy that is believed to provide therapeutic benefit by preventing re-immergence of CD19-positive leukemia cells. One side effect of this activity is prevention of normal B-cell expansion. This therapy is not directly translatable to T-cell leukemia because targeting a T-cell antigen would cause chronic T-cell aplasia and high risk of mortality. We are exploring an alternative CAR T-cell therapy using γδ T cells instead of αβ T cells for the treatment of relapsed/refractory T-cell leukemia because *ex vivo* expanded Vγ9/Vδ2 γδ T cells do not develop a persistent memory phenotype and are predicted to be short lived, with a half-live of less than a few weeks. With respect to T-cell leukemia, we propose γδ T cells can be used in the clinical setting with repeated infusions of CAR-modified γδ T cells as a bridge to stem cell transplantation. We have previously shown a γδ T-cell product candidate can be expanded from PBMCs using a defined serum-free media, which results in a population of up to 90% γδ T cells. Additionally, we can achieve moderate transduction efficiency of these cells with an anti-CD5-CAR lentiviral vector (30-50% modified). Using transduced and non-transduced γδ T cells, we found that transduction using lentiviral vectors encoding an anti-CD5-CAR does not negatively affect their cytotoxic potential. At effector to target ratios of 1:1 and 5:1 in a 4-hour co-culture, 40% and 70% CD5-positive leukemia cell killing, respectively, is routinely observed. Furthermore, at transduction levels of as low as 3% CD5-CAR-modified cells, there is a shift in CD5 expression in the γδ T-cell population from 97% CD5-positive to <50% CD5-positive cells. As we and others have shown with αβ T cells, the down regulation of CD5 limits fratricide among CAR-expressing cells. In addition, to further enhance the effectiveness of CD5-CAR-modified γδ T cells targeting T-cell leukemia, chemokine receptor expression in the expanded γδ T cells was measured by RNAseq. CXCR4, a receptor for CXCL12, showed variable RNA levels in γδ T cells that increased during expansion, with the highest levels detected on day 15 of expansion. CXCL12 is a well described homing signal for T-cell leukemia to the bone marrow. Therefore, high CXCR4 expression on γδ T cells is predicted to increase their anti-tumor effectiveness. Taken together, these data show that γδ T-cell expansion can be optimized for chemokine receptor expression, predicted to enhance T-cell trafficking to the tumor sites, and that these cells can be efficiently engineered to express a CD5-CAR, thereby providing a strong rationale for continued testing of CD5-CAR γδ T cells as a therapy for T-cell leukemia.

119. Overcoming Target Driven Fratricide for CAR-T Cell Therapy

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Chimeric antigen receptor (CAR) provides an approach to putatively target any tumor cell. The success of the approach is largely dependent upon the profile of the target antigen itself where most known tumor antigens are not specific to the tumor. In certain circumstances, the target antigen may be constitutively or transiently expressed on a T cell meaning that the CAR T cell may undergo self-killing or fratricide. A CAR consisting of a fusion of the NKG2D protein with CD3ζ (NKR-2) endows T cells with broad specificity for NKG2D ligands. However, T cells transiently express these ligands during activation and, consequently, NKR-2 T cells undergo fratricide thereby substantially hampering the ability to exploit NKG2D as a therapy. Given the plethora of NKG2D ligands that could be expressed in T cells, genetic editing to eliminate all ligand expression was not feasible. Inclusion of a Phosphoinositol-3-Kinase inhibitor (LY294002) reduced NKG2D expression at the cell surface and blunted the fratricide effect, providing a generic means of generating NKR-2 CAR T cells. Use of the PI3K inhibitor further enhanced NKR-2 driven potency and shifted the cells to a memory phenotype. A target specific approach involving antibody blockade of the NKR-2 CAR itself elicited a further improvement in NKR-2 CAR T cell yield, with a reduction in potency and a change in CD4/CD8 ratio. These factors could successfully be skewed *in vitro* to enhance the potency and change the cell phenotype by delaying the addition of the blocking Ab. Our results demonstrate that target-driven

fratricide can be overcome using different approaches that enable the development of T cell therapies where self-expression of the target ligand is a limiting factor.

120. An Engineered Recombinant Adenovirus Subtype 35 Knob Protein, AD35K++, for Use in Cancer Immunotherapy

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While cancer therapy has seen major advances thanks to the advent of immunotherapy, many challenges still persist in the context of solid tumors. Chimeric antigen receptor (CAR) T-cells have come to prominence in the field of cancer cellular treatment, but its use is hindered in solid tumors due to the immunosuppressive milieu of the tumor, thus resulting in anergy and/or exhaustion of CAR-T cells and eventual resistance to treatment.Adenoviruses are able to bind a large variety of human receptors involved in cell attachment, signalling, and immune modulators. Adenovirus subtype 35 (Ad35) binds the receptor CD46, which is thought to be a complement inhibitor. Recent research has also highlighted CD46's role as an immune (T-cell) modulator, driving inflammatory or suppressive phenotypes of T-cells depending on context and duration. Multiple types of tumors have been found to upregulate expression of CD46 on its surface, presumably as a counter to the innate complement cascade and the tumor-targeting effector T-cells. These properties make CD46 a promising target for treatment of cancers, especially in combination with other therapies, both conventional and cellular.A recombinant, affinity-optimized form of the Ad35 knob protein (termed AD35K++) was developed for use in combination with multiple modes of therapy. Preliminary studies have investigated its efficacy in combination with monoclonal antibody therapeutics such as rituximab in primarily a B-cell malignancy context. A combination of intravenous AD35K++ and intraperitoneal poly I:C, a potent immune adjuvant, resulted in moderately prolonged survival in a CD46-transgenic mouse mammary carcinoma tumor model. AD35K++ also has potential for use in combination with CAR-T cells. In naïve T-cells, AD35K++ was found to drive a pro-inflammatory, effector phenotype with short-term stimulation (~3 hours) which eventually turned into a suppressive phenotype (Tr1) with longer periods of stimulation, as determined by in-vitro cytokine and RNA sequencing assays. Current studies are ongoing to investigate the stimulation of CAR-T cells with AD35K++ to overcome the immunosuppressive tumor microenvironment by driving the T-cells further to an effector phenotype. With the multitude of roles that CD46 plays in the immune system and cancer, AD35K++ is a promising candidate for use in combination with various forms of therapy.

121. Immunomodulatory Therapies Targeting Lck during Gene Therapy May Perturb T Cell Homeostasis

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Introduction: Lymphocyte-specific Src-kinase (Lck) is required for T cell activation (TCA) and generation of an effective T cell response. Immunomodulatory therapies targeting Lck may be a potential strategy to inhibit T cell responses and T cell mediated inflammation during gene therapies. Thus, understanding the role of Lck in T cells is critical for development of Lck-targeting immunomodulatory therapies. Recently, we found that inhibition of Lck in resting human T cells results in enhanced distal T cell receptor (TCR) signaling due to aberrant nuclear translocation of NFAT1 and subsequent IL-2 expression. Inhibitors of the Ca^{2+} dependent phosphatase calcineurin significantly rescued aberrant NFAT1 nuclear translocation suggesting a role for Ca^{2+}/ calcineurin. Here, we studied the mechanism for aberrant NFAT1 nuclear translocation during Lck inhibition in resting human T cells to better understand potential issues that may arise by inhibiting Lck during gene therapy interventions. **Methods**: Human T cell lines (Jurkat and HuT78) and primary human T cells obtained from healthy donors were treated with a selective Lck inhibitor (PP2). Calcium influx was measured by flow cytometry. Lck interaction with Ca^{2+} release activated calcium channel (CRAC) was assessed by confocal microscopy and immunoprecipitation. Phosphorylation of CRAC was assessed by immunoprecipitation and phospho-immunoblotting. **Results:** Resting primary human T cells treated with PP2 demonstrated a dose-dependent increase in calcium influx. Bioinformatics studies identified two tyrosine resides (Y60 and Y115), in the ion channel CRAC, predicted to be phosphorylated by Lck. Structurally, Y60 is located at the N-terminal cytoplasmic tail and Y115 is located on the extracellular loop of the CRAC channel on the plasma membrane. Lck was found to interact with CRAC in human T cells and HEK 293T cells. Furthermore, constitutively active Lck (Y505F) significantly increased CRAC phosphorylation compared to a kinase-dead Lck (K273E). **Conclusions:** These data suggest that Lck binds and phosphorylates the ion channel CRAC. The inhibition of Lck resulted in increased calcium influx and induced aberrant NFAT1 nuclear translocation in T cells suggesting a novel role of Lck in maintaining calcium influx in T cells by CRAC phosphorylation. Current studies are underway to identify the tyrosine residue(s) that are phosphorylated by Lck in CRAC. Furthermore, these data suggest that immunomodulatory therapies targeting Lck may perturb T cell homeostasis by reducing CRAC phosphorylation, and by increasing calcium influx.

122. Establishment of an Animal Toxicity Model for the Prediction of Clinical Utility of Car T Cell Therapies and Technologies

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Chimeric antigen receptor (CAR) T cells have shown unprecedented efficacy in the treatment of hematologic malignancies that are non-responsive to conventional therapies. Yet the translation of this treatment modality to the solid tumor setting remains challenging. Due to the lack of truly tumor-specific antigens, damage of healthy tissue expressing the targeted antigen poses a serious adverse effect. In fact, early attempts in treating solid tumors by CAR T cells resulted in life-threatening or even lethal toxicities as a result of on-target/off-tumor effects. With increasing experience in the

clinic, it has become apparent that the current standard animal testing approach does not allow for proper assessment of CAR T cell-related side effects. In this context, various research questions are still open in regard to the optimal CAR design strategy, as well as the prediction and treatment of those emerging novel toxicities. Aiming for the development of safer CAR T cell therapies, we established an *in vivo* on target/off-tumor CAR T cell toxicity model. The glycolipid SSEA-4 was used as target antigen as its expression has been shown on some normal tissue but a broad array of solid tumors. Due to the correlation of antigen expression between the human and mouse organisms, a preclinical on-target toxicity profiling is possible. By using a human/mouse cross-reactive scFv and varying the spacer region of the receptor, we generated different CAR constructs that showed different *in vivo* toxicity kinetics. Moreover, despite low levels of antigen expression, we saw major alterations in the murine cellular bone marrow composition as well as decreased Sca-1 expression, thus allowing an easier readout of on-target/off-tumor toxicity. This model now enables a thorough comparison of currently described strategies to minimize on-target/off-tumor toxicities and will aid improving the safety profile and clinical outcomes of future CAR T cell therapies.

123. Functional Screening of a B7H6 Specific Chimeric Antigen Receptor (CAR)

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B7H6, a ligand for the NK-activating receptor NKp30, is widely expressed at the surface of transformed cells yet largely absent in healthy tissues. This makes B7H6 an attractive target for a CAR T cell therapy potentially against a broad panel of hematological and solid cancers. CARs are artificial receptors comprising an extracellular antigen-binding region (often a single chain variable fragment (scFv)) fused to an intracellular T-cell activation tail (usually CD3ζ in tandem with one or two costimulatory domain(s)). Here, we report the *in vitro* screening of various B7H6-specific CAR designs differing by either the origin of their targeting moiety (murine versus humanized scFv), the costimulatory signaling modules (either CD28 or 4-1BB as a second generation CAR) or a combination of CD28 and 4-1BB in a third generation CAR context. Primary human T-cell populations expressing the diverse B7H6-specific CAR constructs were compared for in vitro functionality (IFNγ secretion and cytolytic activity in response to challenge with antigen expressing cell lines) to select the best candidate to be evaluated further in in vivo experiments. All anti-B7H6 CAR T-cells led to comparable cellular yields and fold expansion with high viability suggesting that the CAR design has no impact on process parameters. CARs with targeting moiety of murine scFv origin were functionally superior to humanized versions in terms of killing and IFNγ release potentially due to a difference of affinity for B7H6 between the scFv as measured by Biacore. T cells transduced with the second-generation CARs containing the CD28 intracellular domain possessed superior in vitro anti-tumor activity compared to all other constructs. Cryopreservation of these second-generation CAR T-cells did not significantly reduce viability and potency post-thawing. Since the best candidate selected in vitro was a second generation anti-B7H6 CAR with the CD28 moiety, we then studied the therapeutic efficacy of these anti-B7H6 CAR T cells *in vivo* in irradiated NSG mice injected subcutaneously with colorectal Colo 205 tumor cells. Though a variability in tumor growth was observed for Mock T cell-injected mice, mice injected with the second generation of anti-B7H6 CAR T showed a trend to a stabilized tumor growth and to better survival rate compared to control mice. No tumor regression was observed. These results suggest that a second generation anti-B7H6 CARs bearing murine scFv and containing the CD28 intracellular domain could be of therapeutic potential for solid tumors and warrants further investigation for development of a clinical product. These subsequent studies will include target profiling through immunohistochemistry studies assessing B7H6 expression in a wide panel of tumor and normal tissues, and in vivo xenograft models for assessing anti-tumor efficacy of these B7H6 specific-CAR in models of tumors expressing B7H6.

124. Metabolic Response of Natural Killer CD56^{bright} and CD56^{dim} Subsets to Tumor Microenvironment Stresses for Improved Immunotherapies of Solid Tumors

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Tumor microenvironment-induced nutrient deficiencies can cause metabolic reprogramming of innate immune responders, particularly natural killer cells, and discerning these unique metabolic signatures will help to develop clinically effective cancer immunotherapies. Natural killer (NK) cells, defined by expression of CD56^{bright} and CD56^{dim} subsets, have distinct functional differences, and it has been suggested that CD56^{bright} cells display a more robust metabolic response to cytokine-stimulated NK cells. Recent studies have begun to uncover the role of mTORC1 in NK cell metabolism; however, little is known about metabolic hallmarks of NK cells in the tumor microenvironment (TME). TGF-β and extracellular adenosine, both present in the TME, have been shown to suppress the activity of human NK cells, and understanding tumor suppression may help to redirect NK cell function in the TME. Therefore, we are investigating the effect of tumor suppressive signals, including TGF-β and extracellular adenosine, on functional responses of cytokine-stimulated NK cells in normoxic and hypoxic conditions, in order to better understand, regulate, and reprogram NK cell effector function for improved adoptive cancer immunotherapies. For these studies, peripheral blood-derived NK cells were obtained from healthy human donors and isolated by negative selection. Stimulation programs included 200 IU/ml IL-2, 30 ng/ml IL-12+100 ng/ml IL-15, or 100 ng/ml IL-15 with or without TGF-β and/or endogenous adenosine. *Ex vivo*, both mTORC1—measured through s6 and stat5 phosphorylation—and IFNγ were affected in multiple cytokine-stimulating programs due to TGF-β. pstat5 and ps6 appeared to have higher expression in CD56^{bright} cells than CD56^{dim} cells, indicating elevated mTOR metabolic activity. The expression of cytotoxic markers NKG2D, NKp30, NKp44, NKp46, and CXCR3

were impaired in some of the treatment groups, as was the expression of IFNγ. Co-stimulation with IL-12+IL-15 appeared to facilitate a reduction in TGFβ-induced inhibition of IFNγ production. Alongside currently establishing the transcriptional programs and metabolic profiles underlying energy metabolism of cytokine-stimulated NK cells *in vivo* and *in vitro*, we are evaluating NK cell metabolic programs due to hypoxic stress and in the presence of extracellular adenosine to further shed light on the role of adenosine A2A receptors on NK metabolism. Collectively, these insights will uncover information on metabolic pathways that are altered in cancer patients and provide a route to developing immunotherapeutic interventions that can restore altered NK cell function to normalcy following adoptive transfer.

125. CRISPR-Directed Knockout of NRF2 Restores Chemosensitivity in A549 Cell *In Vitro* and *In Vivo*

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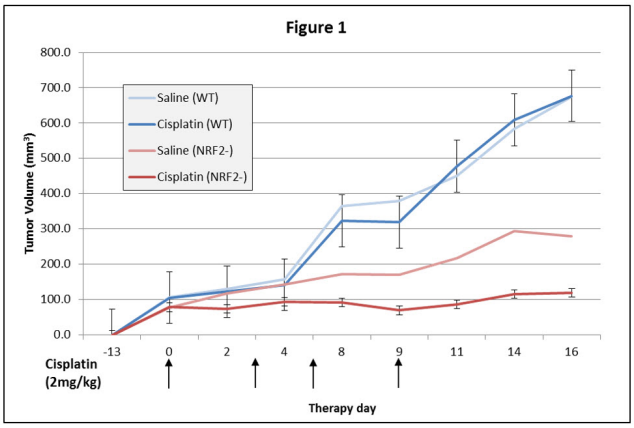

Figure 1. Synergistic effect of CRISPR-directed gene editing and chemotherapy in a Xenograft mouse model.

Introduction. Chemotherapy remains an important option in the treatment of lung cancer but resistance and toxicity can develop with extended treatment**.** Pharmacokinetics and pharmacogenomic studies point to the evolution of drug resistance being centered on the up-regulation of the variety of genes including Nuclear Factor Erythroid 2- Related Factor (NRF2), considered the master regulator of 100-200 target genes involved in cellular responses to oxidative/electrophilic stress. We developed an innovative approach to increase chemosensitivity and potentially boost survival rate by combining Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)-directed gene editing with chemotherapy. The overall strategy was to design and utilize CRISPR/Cas9 to functionally disable the NRF2 gene in lung cancer cells. *We hypothesize that cells bearing a disabled NRF2 gene will exhibit increased sensitivity to Cisplatin as the genes responsible for resistance will not be activated within this genetic background.* ***Results.*** We disabled NRF2 alleles in A549 cells using CRISPR/Cas9 and produced clonal cell lines. The cells were then analyzed for hyper-sensitivity to Cisplatin both *in vitro* and *in vivo***.** To examine the chemosensitivity of the genetically engineered NRF2-deficient A549 cell lines we tested two clones. Clone 1-40 is a heterozygous KO with one intact allele and clone 2-11 is a homozygous KO of NRF2. Wild type, clone 1-40 and a clone 2-11 cells were exposed to increasing dosages of Cisplatin and the viability was tested via MTS. The data show that, as predicted, wild type or unaltered A549 cells are resistant to high dosages of Cisplatin, while the knockout cell lines show a dramatic increase in chemo-sensitivity to Cisplatin in a dose-dependent fashion. Next, a Xenograft mouse model was used to examine tumor response to Cisplatin in the A549 2-11 clone (Figure 1).Wild type A549 cells or NRF2 KO (2-11) clonal cells were implanted onto the back of a nude mouse and allowed to proliferate into a tumor with a volume of approximately 100mm^3. Cisplatin was injected through the tail vein on day 0, 3, 6 and 9 and tumor volume was measured over the course of 16 days. WT A549 tumor growth treated with Cisplatin was not inhibited by the drug, confirming the well-known resistance of A549 cells. NRF2 KO tumors grew at a significantly reduced rate but the most dramatic effect is seen when implanted NRF2-KO cells were treated with Cisplatin. In this case, tumor growth was completely arrested throughout the course of the experiment. ***Conclusion.*** These data suggest that a synergistic relationship exists between CRISPR-directed NRF2 gene disruption and chemotherapy for significantly enhanced killing of NSCLC cells in a Xenograft mouse model

126. Design and Functional Analysis of Fn3-Based Bi-Specific Cars

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Despite the spectacular efficacy of CART cell therapy in treating various B-cell malignancies, antigen escape is a pressing problem for mono-specific CARs and the success of this approach in solid cancers is still modest, at best. Bi-specific CAR T cells offer the opportunity of mitigating antigen escape and coping with tumor cell heterogeneity. Yet, the design of such bi-specific single-chain CARs compatible with the unique spatial arrangement of the epitopes to be targeted is known to be complicated. We asked whether the use of alternative and more compact antigen-recognition moieties, such as Fn3s (or adnectins), would produce functional bi-specific CARs. To address this question, we combined several model Fn3-based modules recognizing VEGFR2, CEA, and IGF-1R and designed lentiviral constructs encoding bi-specific CARs with distinct hinge and linker sequences. To this end, the biFnCARs obtained were expressed in Jurkat cells and tested in activation assays wherein effector cells were incubated with the cells expressing one or two target proteins. Of all the combinations, only few biFnCARs were truly bi-specific, whereas the majority of the constructs displayed little to no synergy in binding of the Fn3 domains to their cognate targets. Thus, in our experience the design of Fn3-based bi-specific CARs does not appear advantageous to that of the scFv-based bi-specific CARs and similarly requires extensive structural optimization. This study was supported by the grant from the Russian Science Foundation # 16-14-10237.

Cancer - Targeted Gene & Cell Therapy I

127. Improving Efficacy of CAR T Cells through CRISPR/Cas9 Mediated Knockout of TGFBR2

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CD19-directed chimeric antigen receptor (CAR) T cells have shown efficacy in the treatment of certain B cell malignancies resulting in the recent FDA approval of two CD19-CAR T cell therapies. As this class of therapies matures and other malignancies are targeted by engineered T cells, the immunosuppressive nature of certain tumor microenvironments may blunt the anti-tumor activity of certain adoptively transferred T cells. The anti-inflammatory cytokine, transforming growth factor β (TGFβ) is elevated in the tumor microenvironment for a variety of tumors and is a potent suppressor of T cell proliferation and inhibitor of effector function. The suppressive effect of TGFβ involves signaling through TGFβ Receptor 2 (TGFβRII), which is a transmembrane protein that dimerizes with TGFβRI when bound to TGFβ, an event that initiates a signaling cascade resulting in inhibition of cell proliferation and changes in T cell cytokine production. To generate T cells that are resistant to the suppressive effects of TGFβ, we evaluated several RNA-guided CRISPR (clustered regularly interspaced short palindrome repeats)-associated nuclease Cas9 sites in the TGFBRII locus to determine the most effective means of generating loss-of-function mutations. Using an in silico off target prediction software, gRNAs with favorable specificity profiles were chosen and single guide RNA/Cas9 complexed ribonucleoproteins (RNPs) were screened in primary activated T cells. On-target editing efficiency was evaluated by next generation sequencing of pooled PCR amplicons that spanned the predicted cut sites. The gRNAs that resulted in the most active RNPs were further characterized and validated by evaluating the ability of edited cells to become insensitive to TGFβ-mediated suppression. To confirm that TGFβRII editing could improve the functionality of CAR T cells, RNP delivery was paired with lentiviral transduction of various CAR constructs having specificity to different antigens, in order to generate CAR T/TGFβRII edited cells. These cells, regardless of the antigen they were targeting, were shown to be insensitive to TGFβ-mediated inhibition of proliferation, cell cytotoxicity and cytokine production. Furthermore, these cells had improved effector function against target expressing cell lines in a TGFβ rich environment *in vitro*. Lastly, TGFβ editing prevented the development of a TGFβ-induced gene expression phenotype in a NSG mouse xenograft model. These data suggest that utilization of TGFβRII gene editing using CRISPR-Cas9 may provide an effective way of overcoming the TGFβ-mediated suppression of engineered T cell therapy, and this platform could be broadly applicable across different CAR products.

128. Nanoparticle-Mediated Gene Therapy against DANCR Suppresses TNBC by Targeting Multiple Oncogenic Pathways

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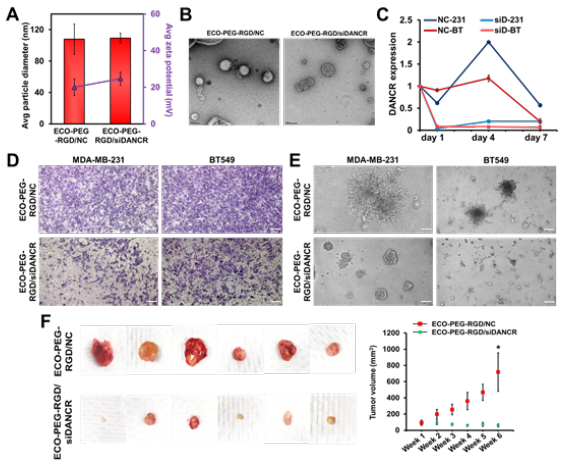
Long non-coding RNAs (lncRs) are a biochemically versatile species that exert multilevel gene regulation at the epigenetic, transcriptional, and translational level in a tissue- and disease-specific manner. In heterogenous and complex malignancies, such as triple-negative breast cancer (TNBC), numerous aberrantly expressed lncRs are known to significantly impact clinical outcomes by influencing global gene signatures. Here, we demonstrate effective therapeutic efficacy by successful and efficient nanoparticle-mediated silencing of the oncogenic lncR, DANCR (differentiation antagonizing lncR) in a TNBC mouse model. We show that DANCR is significantly overexpressed in TNBC cells and patient tissues, compared to their normal counterparts. Since DANCR cannot be targeted by conventional chemotherapy, we implemented the multifunctional cationic lipid carrier, ECO, to deliver therapeutic siRNA against DANCR in TNBC models. Stable self-assembly ECO/siDANCR nanoparticles were modified with PEG conjugated with the RGD peptide to improve biocompatibility and targeted delivery to cancer cells, respectively. ECO-PEG-RGD/NC nanoparticles with non-specific siRNA were used as negative control (NC). The formulations were characterized for size, zeta potential, siRNA entrapment, and morphology. MDA-MB-231 and BT549 cells transfected with the therapeutic ECO-PEG-RGD/siDANCR nanoparticles exhibited 80-90% knockdown in the expression of DANCR for up to 7 days, indicating efficient intracellular siRNA delivery and sustained target silencing. Compared to the NC, the ECO-PEG-RGD/siDANCR nanoparticles facilitated excellent therapeutic efficacy, reflected by the significant inhibition in the migratory and invasive abilities of the cancer cells through Matrigel-coated Transwell inserts and scratch-wounds, coupled with a significant decrease in cancer cell viability, proliferation, and 3D Matrigel tumor spheroid formation. Finally, systemic administration of the therapeutic nanoparticles in nude mice implanted with MDA-MB-231 xenografts demonstrated significant reduction in tumor size and volume, with negligible effects on vital organs, underscoring the safety and efficacy of the tested therapy. At the molecular level, silencing of DANCR was found to mediate its functional effects by suppression of PRC2 complex-mediated H3K27 trimethylation and by downregulating EMT (ZEB1 and N-cadherin), Wnt (β-catenin), and anti-apoptotic (Survivin) signaling markers. This is the first study that demonstrates effective *in vivo* ECO/siRNA-mediated lncR targeting for TNBC therapy. Our results represent the first successful step towards the development of a safe and effective gene delivery platform to target cancer-associated lncRs for the treatment of TNBC and various other cancers.



ECO-PEG-RGD/siDANCR and NC nanoparticles were tested for **A.** size and zeta potential, **B.** morphology and **C.** sustained silencing. Therapeutic formulation inhibits **D.** migration and **E.** 3D tumor spheroid growth of TNBC cells. **F.** Systemic nanoparticle therapy of TNBC xenografts results in significant decrease in tumor burden.

129. Glypican-3-Specific CAR NKT Cells Armored with IL-15 Exhibit Potent and Sustained Anti-Tumor Activity against Hepatocellular Carcinoma

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Poor clinical outcomes of hepatocellular carcinoma (HCC) patients highlight the need for novel therapeutic approaches to fight this malignancy. Immunotherapeutic targeting of glypican-3 (GPC3) is an attractive option, as this antigen is highly expressed on HCC tumor cells but not healthy, mature tissues. Two recent phase 1 clinical studies have shown that GPC3-targeted therapies are well-tolerated and produce transient disease control. A promising immunotherapeutic approach is to express a chimeric antigen receptor (CAR) in effector lymphocytes, combining the specificity of a monoclonal antibody with the long-term persistence, active bio-distribution, and cytolytic function of immune cells. CAR T cells mediate sustained tumor eradication in patients with CD19+ malignancies, but have shown limited success against solid tumors including HCC. Vα24-invariant natural killer T cells (NKTs) are promising effectors for expression of tumor-specific CARs due to their natural anti-tumor properties and ability to infiltrate and persist in solid tumors including HCC. More recently, the homeostatic cytokine IL-15 has been shown to promote the anti-tumor activity of NKTs in the hypoxic solid tumor microenvironment. We generated GPC3-specific second-generation CAR constructs incorporating the 4-1BB co-stimulatory endodomain with or without IL-15 (15.GPC3-CAR or GPC3-CAR, respectively), and hypothesized that co-expression of IL-15 with the GPC3-CAR would promote NKT cell expansion, persistence, and potent anti-tumor activity in HCC-bearing mice. We found that all CAR constructs enabled NKTs to specifically and effectively kill HCC cells *in vitro* as measured by standard ^{51}Cr release assays. Multiplex cytokine analysis revealed that CAR NKTs secreted a range of effector cytokines including GM-CSF, IFNγ, and TNFα in response to *in vitro* stimulation with GPC3+ HCC cells. Compared to the GPC3-CAR construct alone, co-expression of IL-15 significantly enhanced the persistence of GPC3-CAR NKTs (Fig 1, One-way ANOVA; $p<0.05$) in NSG mice bearing HCC xenografts. In an aggressive HCC xenograft model, infusion of GPC3-CAR NKTs produced robust anti-tumor responses, which were further enhanced by co-expression of IL-15 such that the majority of animals receiving 15.GPC3-CAR NKTs attained curative responses (Figure 2; Controls vs GPC3-CAR NKTs $p<0.009$, GPC3-CAR NKTs vs 15.GPC3-CAR NKTs $p< 0.05$; Mantel-Cox analysis). Thus, the combination of 4-1BB co-stimulation and IL-15 pro-survival signaling provided by the 15.GPC3-CAR construct promotes GPC3-CAR NKT cell *in vivo* persistence and long-term tumor control in a pre-clinical HCC model. These results warrant further development and clinical testing of GPC3-targeted CAR NKTs in HCC patients.

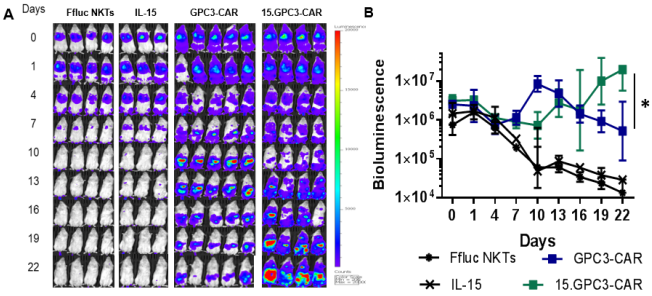

Figure 1. Expansion of GPC3-CAR NKTs in vivo: Human NKTs were transduced with Ffluc alone or with IL-15, GPC3-CAR or with a construct co-expressing IL15 and GPC3-CAR. NKT expansion and persistence was monitored with bioluminescence imaging at the indicated time points. (A) NKT cell bioluminescence images. (B) Bioluminescence counts graphed over time. Day 22 persistence was compared with ANOVA. *p<0.05

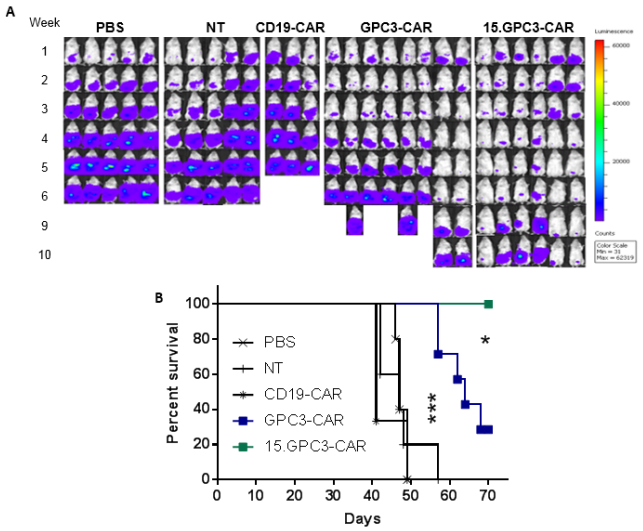

Figure 2. NKT cells co-expressing IL-15 and GPC3-CAR have superior anti-tumor activity against HCC cells. NSG mouse were IP injected with $2x10^6$ Ffluc+ Huh-7 HCC cells at week 0. $6x10^6$ CAR+ NKT cells were IV injected at Week 1 (Non-transduced (NT), CD19-CAR and PBS injections served as controls. (A) Tumor bioluminescence images at indicated time-points (B) Kaplan-Meier survival of tumor bearing mice. Log-rank test * p<0.05, ** p<0.01, *** p<0.001.

130. Universal CAR T-Cells Targeting CS1 (UCARTCS1) for the Treatment of Multiple Myeloma

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CS1 (also called SLAMF7) is highly expressed on multiple myeloma (MM) tumor cells and has limited expression in a subset of hematopoietic cells among normal tissues, making it a rational target for chimeric antigen receptor (CAR) T-cell therapy. Currently, most CAR T-cell products are generated from autologous T-cells, which can be a major limitation logistically for lymphopenic and critically ill patients. Therefore, we have designed allogeneic "off-the-shelf" engineered CAR T-cells (derived from normal healthy donor mononuclear cells), which contain an inactivation of the *TCRα constant* (*TRAC*) gene using TALEN® gene-editing technology. The inactivation of the *TRAC* gene prevents cell surface expression of the T-cell receptor (TCR) αβ complex, eliminating TCR-mediated recognition of histocompatibility antigens that can lead to GvHD. As CS1 is expressed on activated $CD8^+$ T-cells, the *CS1* gene is also knocked-out using TALEN® in order to avoid fratricide of CS1-specific CAR^+ T-cells. The inactivation of the *CS1* gene in T-cells prior to the introduction of the anti-CS1 CAR construct effectively prevents the elimination of $CD8^+$ CAR^+ T-cells and maintains a naïve phenotype in the CAR^+ T-cell population. *In vitro* cytotoxic assays against MM cell lines show that gene-editing (CS1 KO) of CAR^+ T-cells induces higher anti-tumor activity compared to non gene-edited CAR^+ T-cells. In addition, cytotoxicity assays show that UCARTCS1 did not induce significant lysis of normal donor peripheral blood mononuclear cells or $CD34^+$ hematopoietic cells from bone marrow aspirates of healthy donors. Studies examining *in vivo* anti-tumor efficacy have been performed. NSG mice were intravenously injected at Day -10 with $5x10^6$ MM1.S tumor cells expressing the luciferase. 10 days later, when the tumor had been established, mice were treated intravenously with a single injection at Day 0 or two injections at Day 0 and Day 25 of 3 or $10x10^6$ UCARTCS1 cells or left untreated (vehicle injection). Mice injected with vehicle displayed a gradual increase in their tumor burden by *in vivo* imaging whereas mice treated with UCARTCS1 cells control the development of the tumor. Furthermore, all mice treated twice with 10 x 10^6 UCARTCS1 showed a very efficient and sustained control of the tumor progression with undetectable M protein levels in their serum at Day 67 and with all mice alive up to 100 days post adoptive transfer. Finally, UCARTCS1 cells have been shown to specifically target and lyse primary MM tumor cells *in vitro* and *in vivo* using primary MM xenografts in human fetal bone implanted into NSG mice (NSG-hu). Our results support further development and testing of this universal "off-the-shelf" allogeneic CS1-specific CAR-T product in patients with MM with potential re-administration of UCARTCS1 cells in patients.

131. Development of an *In Vitro* Cynomolgus Macaque Allogeneic CAR T Cell Platform: Working towards a Reliable *In Vivo* Allogeneic Model to Assess Safety and Efficacy

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Chimeric antigen receptor (CAR) T cell therapies have recently been approved for commercialization and are proving to be transformative therapies in the fight against hematological malignancies. Allogeneic, or "off-the-shelf", CAR T cells generated from healthy donors allow for immediate treatment of large cohorts of patients, and may reduce the risk of manufacture failure associated with bespoke autologous therapies. As treatment modalities attempt to target other hematological and solid malignancies, there is a need for a robust and reliable pre-clinical animal model to validate the safety of novel targets potentially expressed on healthy tissues, as well as, to evaluate the potential graft-versus-host disease (GvHD) risk of an allogeneic CAR T cell therapy. Using cynomolgus macaques as an animal model, we have developed a reliable *in vitro* platform that allows for the generation and expansion of large numbers of cyno CAR T cells. First, by using an optimized cyno T cell activation and expansion protocol, followed by a retronectin-based gamma-retroviral dual transduction stage, optimal and stable expression of several CAR T constructs was achieved. Next, by optimizing CAR T cell expansion and formulating conditions, cyno CAR T cells were generated with improved CD4:CD8 ratios, minimal tonic signaling and enriched for early-differentiated $CD62L^+$ T cells. Cyno CAR T cells manufactured with this optimized protocol display minimal activation in steady state conditions, and are readily activated by co-culture with target-bearing cells. An anti-CD19 CAR, based on FMC63 (human-specific), and an anti-CD20 CAR, based on Ofatumumab (Human/Cyno cross-reactive), were used to generate Cyno 4-1BBz-based CAR T cells as proof-of-concept models. Large-scale batches of anti-CD19 and CD20 cyno CAR T cells were readily manufactured in our optimized process conditions and displayed optimal transduction and phenotype. Furthermore, these cyno CAR T cells displayed high post-thaw functionality against Raji and Daudi $CD19^+$/$CD20^+$ lymphoma target cells. Additionally, we have successfully achieved inactivation of the T cell receptor (TCR) complex through TALEN® induced gene-editing of the *T Cell Receptor Alpha* (*TRAC*) locus in cyno CAR T cells, with knock-out efficiencies up to 75% with minimal impact on cell viability and expansion capacity. After depletion of residual TCR^+ cells, 97% of the CAR T cell culture was $CD3^-$ by flow cytometry. On-going efforts are directed at testing the *in vivo* efficacy of cyno CAR T cells in Raji-bearing NSG mice as a gate-keeping experiment that will endorse moving into autologous and allogeneic proof-of-concept studies in non-human primates. Overall these *in vitro* results demonstrate that cyno CAR T cells are a reliable *in vitro* platform to enable allogeneic CAR T cell safety studies in non-human primates.

132. Delivery of CIB1-siRNA Using Macrophages for Breast Cancer Treatment

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Macrophages represent a significant proportion of tumor mass, particularly in breast, prostate, and colon cancers and as such presents a novel opportunity for use as a drug/gene delivery vehicle. Conventional wisdom has dictated that macrophages, a professional phagocytic cell, impedes drug/gene delivery by ingesting local therapeutic nanoparticles. However, recent discoveries are revealing that this is not accurate. Macrophages act as drug reservoirs in the tumor microenvironment, engulfing therapeutic nanoparticles and subsequently releasing the drug contents. Furthermore, macrophages when presented with exogenous plasmid DNA can transfer that DNA to muscle cells *in vivo* and that this mechanism is intensified under ischemic conditions. As the role for macrophages in existing drug delivery emerges, there is a potential to synchronize this behavior to deliver therapeutics in an intentional capacity to the tumor microenvironment. Here we developed an *in vitro* high throughput tumor spheres platform and an *in vivo* orthotopic mouse model to study the ability of macrophage to deliver functional siRNA to treat breast cancer which has one of the highest densities of macrophage infiltration, We co-cultured human breast cancer cell lines MDA-MB-468 and IC 21 macrophages to create tumor spheres. IC 21 macrophages were transfected with CIB1-siRNA labeled with Cy5.5 (IC21-CIB1siRNA), which is known to reduce proliferation, migration, and induce apoptosis in triple negative breast cancer (TNBC) cells. Using live-cell imaging platforms we characterized macrophage transfer of CIB1-siRNA to cancer cells, and measured the efficacy via parameters such as growth of the tumor spheroid (Figure 1). Furthermore, we adoptively transferred IC21-CIB1siRNA into orthotopic MDA-MB-468 tumors to test the pharmacokinetics of macrophage distribution and the pharmacodynamic effect of siRNA transfer on tumor progression. Upon harvesting tumor tissue into single cells, we used FACS analysis to quantify the percentage and unique population of cells containing siRNA as well as the percentage of apoptosing (Annexin-V-positive) cells to measure activity of the siRNA delivery. Understanding the intratumoral conditions under which gene transfer is successful and results in potent anti-tumor effects could yield valuable information about the use of macrophages for gene therapy. The results found here could also be applied to the use of other immune cells as drug delivery carriers.

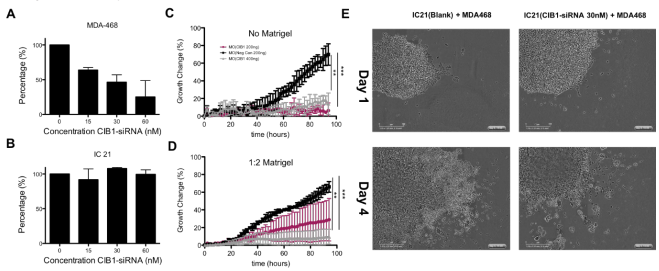

Figure 3 Macrophages loaded with CIB1-siRNA results in decreased growth of breast cancer cells. A) Cytotoxicity measurement of MDA-468 breast cancer cells after transfection with various concentrations of CIB1-siRNA. B) Growth curve of IC21 macrophages after transfection with negative control- siRNA and CIB1-siRNA. Percentage growth change in 3D tumorspheroids with C) no matrigel and D) 1:2 matrigel. E) Images of IC21(Blank) + MDA-468 (left) and IC21(CIB1-siRNA 30nM) + MDA-468 (right) at Day 1 (top) and Day 4 (bottom). n=3 for all samples. One-way ANOVA analysis performed with Tukey post-hoc test. **p<0.01 ***p<0.001

133. Dose Dependent Exogenous Regulation of Membrane Bound Interleukin-15-Interlukin-15 Receptor Alpha for Adoptive T-Cell Therapy

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Adoptive cell therapy utilizing chimeric antigen receptor (CAR) modified T cells has shown remarkable clinical success in the treatment of B cell malignancies. However, in solid tumors, CAR-T cells have been unable to achieve the same level of efficacy as they have in B-cell leukemias, possibly due to the immunosuppressive microenvironment, clonal heterogeneity of solid tumors and limited access of T-cells into the tumors. The ability of CAR-T cells to traffic to tumor sites provides an opportunity for selective co-delivery of cargo that has the capability of enhancing CAR-T cell activity at the site of the tumor. Membrane bound Interleukin-15-Interleukin 15 receptor alpha (mbIL-15) has previously been shown to enhance CAR-T activity, yet unregulated expression can compromise safety and efficacy. Obsidian Therapeutics is developing a novel platform for regulation of transgenes at the protein stability level using destabilizing domains (DD). The DD platform enables dose dependent and reversible regulation, of transgene through administration of clinically approved small molecules. We have used a DD derived from *E. Coli* Dihydrofolate (ecDHFR), which is inherently unstable and degraded but can be stabilized by the addition of Trimethoprim (TMP). Fusion of ecDHFR DD to mbIL-15, confers instability to the fusion protein, which is rapidly degraded in the absence of ligand. In the presence of TMP, mbIL15-ecDHFR protein is stabilized, allowing expression at the desired level in a dose dependent manner. We describe here the successful implementation of the DD technology in engineered primary human T cells and show TMP-controlled regulation of DD modified mbIL-15 protein expression both *in vitro* and *in vivo* in mouse models. These data demonstrate the feasibility of exogenous control over transgene derived protein expression in primary human T cells for the development of next generation CAR-T cell products with enhanced efficacy and more favorable safety profiles.

134. Genome Editing for Irreversible Tumoricidal Gene Therapy

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The RNA-guided endonuclease CRISPR/Cas9 system has emerged as a highly efficient genome-editing platform, while recombinant adeno-associated virus (AAV)-based vectors provide versatile tools for widespread gene delivery in vivo. Cas9 from Staphylococcus aureus (saCas9) allows generation of high titer, single AAV vectors carrying both saCas9- and guide RNA-expression cassettes. Although various anti-cancer strategies have shown some promise in the treatment of human malignancies, emergence of treatment-resistant cells has been a formidable challenge. Our central hypothesis is that the highly efficient genome editing technology may offer a novel

tumoricidal strategy to induce irreversible fatal genome modifications in cancer cells. To test this, we employed the AAV-saCas9-gRNA vectors and assessed two approaches including (i) targeted disruption of an essential cell-cycle gene, CDK1, for anti-proliferation and (ii) targeting high-copy repeats in human genome (HERV-9, ~1000 copies/cell; HERV-K, 200 copies/cell) for induced genome fragmentation. Both strategies strongly blocked proliferation of cancer cell lines and induced rapid cell death in vitro. However, the CDK1-targeted strategy frequently led to emergence of escape mutant cells with 6 nucleotide deletions in the gRNA-targeted site. In contrast, induced genome-fragmentation strategies were more effective than targeting a single cell-cycle gene. In a mouse xenograft model, repeated IT administrations of the HERV-targeted AAV vector significantly delayed the growth of HeLa cells. Thus, the CRISPR-mediated genome editing technology can provide unique anti-cancer strategies.

135. Novel Heterogeneous Glioblastoma Models to Optimize Human Tumoricidal Stem Cell Therapy

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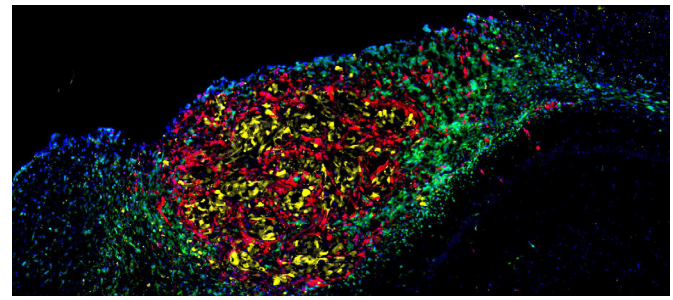
Background: The heterogeneity and invasiveness of glioblastoma (GBM) make this tumor especially difficult to treat. Preclinical models that display important aspects of this disease are needed to guide the development of new therapies. In this study, we used a combination of established and patient-derived GBM cell lines, novel living organotypic brain slices, and heterogeneous *in vivo* models to more accurately recapitulate the hallmarks of clinical GBM. We then utilized these novel models to explore the impact of tumor heterogeneity on induced tumoricidal stem cell therapy, a promising new treatment for GBM. Methods: To initially quantify the growth, migration, and drug sensitivity of multiple GBM cell types, a panel of primary and established GBM cells were cultured alone or in combination on live organotypic brain slices and tracked using serial fluorescence imaging. Combinations of these cells were then implanted orthotopically into nude mice to generate two heterogeneous tumor models. Fluorescence imaging, IHC staining, and MRI were used to analyze tumor morphology. To investigate therapy, human tumoricidal stem cells expressing the anti-tumor agents TRAIL, thymidine kinase/ganciclovir, or a combination therapy (TRAIL + TK/GCV) were used the treat the heterogeneous tumors. Kinetic imaging and post-mortem fluorescence/immunohistochemical analysis of brain sections were used to track differential responses to mono- or combination stem cell therapy. Results: *In vitro* tumor cell analysis on brain slices showed that certain mixtures of cells segregated into solid cores surrounded by invasive borders, while other combinations created more evenly mixed heterogeneous tumors. In vivo, the heterogeneous models recapitulated several aspects of clinical GBM, including solid/invasive regions, heterogeneous HIF1-α expression, and enhancing/non-enhancing regions on MRI. Again utilizing the living brain slice cultures, tumors displayed a spectrum response to stem cell monotherapy, with certain populations showing nearly 100% cell death while other cell types exhibited significant resistance to treatment. *In vivo*, we found that tumoricidal stem cell mono-therapy with either TRAIL or TK/GCV significantly delayed tumor growth and extended survival compared to control-treated mice, with TRAIL-TK/GCV combination therapy further improving treatment responses and extending survival more effectively. Post-mortem analysis also showed that the tumors which recurred following multi-agent therapy were dramatically altered in both the cellular composition and distribution pattern of tumor cells compared to tumors treated with stem cell mono-therapy. Conclusions: These novel heterogeneous models allow optimization of a promising GBM therapy by challenging the therapy to overcome many hallmarks of patient GBM. Combination therapy against these aggressive and invasive models showed significant success and can be further improved using these models to test variations in treatment schedule and route of administration.



136. p53-tBid Hybrids Hijack the Mitochondria for Ovarian Cancer Gene Therapy

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p53 is known as 'The Guardian of the Genome' for its role as a transcription factor that regulates growth cycle arrest, DNA repair and apoptosis. Despite its notorious reputation and inactivation in most cancers, introduction of wild type p53 (WT-p53) has failed as a gene therapy. WT-p53 has failed a number of clinical trials in the US, including trials for patients with ovarian cancer. Dominant negative inhibition of exogenous WT-p53 by endogenous mutant p53 (resulting in an inactive tetrameric transcription factor) may be in part responsible for the failing of such therapies. In order to overcome dominant negative inhibition we sought to exploit an alternative transcription independent role of p53 as a pro apoptotic mitochondrial protein. p53 localizes to the mitochondria in response to cellular stress. There it interacts with pro and anti-apoptotic Bcl-2 proteins to promote mitochondrial out membrane permeabilization that leads to intrinsic apoptosis. In previously published work, our lab has shown that targeting p53 to the mitochondria by fusing a mitochondrial targeting signal to p53's C-terminus can induce apoptosis more potently than WT-p53 in some ovarian cancer cell lines. We sought to further increase the strength of the mitochondrial targeted p53 fusion by fusing the C-terminus of p53 to a pro-apoptotic mitochondrial targeted Bcl-2 protein. Activation of apoptosis through manipulating the balance of pro and anti-apoptotic Bcl-2 family proteins has been attempted with BH3 inhibitors of anti-apoptotic proteins, and introduction of pro-apoptotic Bcl-2 proteins via gene therapy. We chose BH3-interacting domain death agonist

(BID). BID is constitutively expressed and localized in the cytosol. When cleaved by caspase 8 during FAS mediated cell death, the c-terminal truncation product (tBid) localizes to the mitochondria and acts as a potent inducer of apoptosis. tBID can directly interact with Bax to induce oligomerization, mitochondrial outer membrane permeabilization, and eventually apoptosis; however, tBid can be inhibited by interactions with the anti-apoptotic Bcl-2 family proteins. In order to harness the killing power of p53 and tBid simultaneously, we created a novel p53-tBid fusion protein connected by a flexible 3x GGGGS linker. Using fluorescent microscopy at 60x magnification, we show that EGFP-p53-tBid co-localizes with the mitochondria (MitoTracker™). Once there, we hypothesize that apoptosis is induced through both p53 and tBid dependent mechanisms. Flow cytometry analysis of tetramethylrhodamine ethyl ester staining for apoptotic cells shows that p53-tBid is significantly more effective than WT-p53 gene therapy in human ovarian cancer cell lines with varying p53 status: Kuramochi (dominant negative, structural p53 mutant), and SKOV-3 (p53 null). The efficacy of WT-p53 is increased 7 fold when fused to tBid in Kuramochi cells and 2.5 fold in SKOV-3 cells. Because p53 can bind to and inhibit anti-apoptotic Bcl-2 proteins, we hypothesize that the killing activity of the fusion will also surpass that of wild type tBid in cell lines and preclinical models that overexpress Bcl-2 anti-apoptotic proteins. If this construct can overcome the shortcomings of WT-p53 gene therapy and Bcl-2 anti-apoptotic protein overexpression, it can be a viable candidate to treat pre-treated drug resistant ovarian cancer tumors, which often exhibit increased expression of Bcl-2 anti-apoptotic proteins compared to untreated tumors.

137. Selective Excision of an EGFR Mutant Allele in Lung Cancer by CRISPR/Cas9 System Leads to Tumor Growth Inhibition

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Approximately 15% of non-small cell lung cancer cases are associated with a mutation in the epidermal growth factor receptor (EGFR) gene, which plays a critical role in tumor progression. With the goal of treating mutated EGFR-mediated lung cancer, we demonstrate the use of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) system to discriminate between the oncogenic mutant and wild-type EGFR alleles and eliminate the carcinogenic mutant EGFR allele with high accuracy. We targeted an EGFR oncogene harboring a single-nucleotide missense mutation (CTG>CGG) that generates a protospacer-adjacent motif sequence recognized by the CRISPR/Cas9 derived from S. pyogenes. Co-delivery of Cas9 and an EGFR mutation-specific single-guide RNA via adenovirus resulted in precise disruption at the oncogenic mutation site with high specificity. Furthermore, this CRISPR/Cas9-mediated mutant allele disruption led to significantly enhanced cancer cell killing and reduced tumor size in a xenograft mouse model of human lung cancer. Taken together, these results indicate that targeting an oncogenic mutation using CRISPR/Cas9 offers a powerful genome editing strategy to disrupt oncogenic mutations to treat cancers; similar strategies could be used to treat other mutation-associated diseases.

138. Modeling Pediatric AML Associated Mutations with CRISPR Mediated Gene Editing

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Acute Myeloid Leukemia (AML) is a disorder of the hematopoietic progenitor cell. Pediatric AML is a rare disease with only ~500 children diagnosed each year. Although prognosis has improved over the last few decades, relapse is still a major concern accounting new therapeutic targets that reverse the negative prognosis encountered in infants and adults with leukemia. Genetic engineering tools can be used to help in the discovery of new anti-leukemic drugs by enabling the creation of modified cell lines that recapitulate the molecular events that precede leukemogenesis. We have successfully utilized a CRISPR-directed gene editing to catalyze the exchange of the appropriate segments of chromosome 4 and for more than half of the pediatric leukemia deaths. Thus, more relevant cell-based models are needed to enable the discovery of chromosome 11 to generate a cell line bearing a (4:11)(q21:q23) translocation (Figure 1). This chromosomal exchange appears in a series of pediatric leukemias that exhibit a poor outcome. This cell line can now be used for the screening of appropriate compounds designed to inhibit leukemogenesis in children bearing this chromosomal translocation. Our next goal is to develop a sensitive and a reliable system to detect and monitor leukemic clones with drug-resistant FLT3 mutations. We predict that the sequential acquisition of mutations de-sensitizes leukemic cells to tyrosine kinase inhibitors. We will develop a system through the construction of the plasmids expressing the FLT3 ITD bearing specific point mutations to determine the functional impact of FLT3 leukemic mutations. We will further examine differential sensitivity to tyrosine kinase inhibitors (Figure 2).

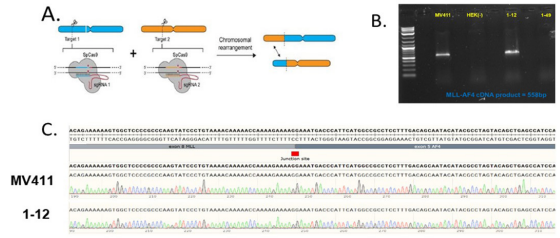
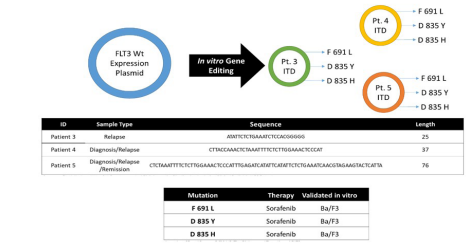


Figure 1. CRISPR/Cas9 Mediated Chromosomal Translocation. (A) The translocation strategy. (B) RT-PCR analysis of clone 1-12 for primary translocation MLL-AF4. (C) Sequence chromatogram of the detected MLL-AF4 breakpoint junction from cells in which Cas9 and both KMT2A and AFF1 sgRNAs are expressed.



ID	Sample Type	Sequence	Length
Patient 3	Relapse	ATATTCTCTGAAATCTCCACGGGGGG	25
Patient 4	Diagnosis/Relapse	CTTACCAAACTCTAAATTTCTCTTGGAAACTCCCAT	37
Patient 5	Diagnosis/Relapse /Remission	CTCTAAATTTTCTCTTGGAAACTCCCATTTGAGATCATATTCATATTCTCTGAAATCAACGTAGAAGTACTCATTA	76

Mutation	Therapy	Validated in vitro
F 691 L	Sorafenib	Ba/F3
D 835 Y	Sorafenib	Ba/F3
D 835 H	Sorafenib	Ba/F3

Figure 2. Construction of the plasmids that will express the FLT3 ITD along with the different point mutation.

139. Targeted Delivery of Chemotherapeutic Agents towards Human Adenocarcinoma Cell Lines via Graphene Quantum Dot's

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Graphene is a unique two-dimensional material possessing exceptional optical, thermal, mechanical and electronic properties. Its charge carriers mimic quantum particles primarily due to features including the quantum confinement and edge effect, this coupled with its seemingly immense potential across various applications is currently generating much interest. These unique properties are being explored for applications including advanced electronics, energy technology and composite materials. Its applications in the biomedical field is of special interest, with drug delivery and cellular imaging at the apex of that list. In respect to drug delivery, most available anticancer agents provide some benefit, however are rapidly metabolized in the body after administration, this coupled with failure to reach optimum concentrations at site of the tumor effectively reduces their efficacy. In addition, they produce unpleasant and severe side effects. Hence, the need for a safe and effective drug delivery system that will ensure sustained and controlled drug release to the desired tumor site. In this study, Graphene quantum dots (GQD's) were chemically synthesized via a modified hummers method, followed by functionalization with the cationic polymer chitosan for delivery of the anticancer agents 5-fluorouracil (5-FU) and leucovorin. Respective nanocomposites were conjugated with folate, as a targeting moiety which has been shown to bind to folate receptors expressed on the surface of breast and cervical cancer cell lines. All nanoparticles and nanocomposites were fully characterized using UV-vis spectroscopy, ICP, FTIR, SEM, AFM, XRD and Nanoparticle Tracking Analysis. The anti-oxidant ability of the nanocomposites will be determined through assays including DPPH scavenging, H_2O_2 scavenging, Nitric oxide scavenging and Ferrous ion (fe^{2+}) chelating activity. The ability of the nanocomposites to encapsulate, deliver and release the anticancer agents under the appropriate conditions was investigated, thus determining the suitability of GQD's as a delivery vector. In addition, studies involving mucoadhesion, drug binding, molecular docking, cellular uptake studies and drug release over a 72-hour period, were carried out. Previous studies have demonstrated that GQD's alone exhibit low toxicity, therefore all nanoparticles and nanocomposites will be investigated for biological activity using two *in vitro* cytotoxicity assays i.e MTT and SRB across two human cancer cell lines, viz. MCF-7, KB-11 and a non-cancer control cell line, HEK293. Apoptosis assays will be conducted across all three cell lines to determine the mechanism of cell death. The ability of GQD's to act as a delivery vector has been demonstrated previously, this study involves the targeting of a unique combination of anticancer agents functionalized to GQD's. This study aims to display a clear specificity of the nanocomplexes towards the cancer cell lines with little or no significant toxicity toward the non-cancer cell line. Thereby highlighting the immense potential of this nanocomposite in providing safe and efficient drug delivery with limited side effects.
Keywords: Nanotechnology, Graphene quantum dots, Drug delivery, Targeting, Folate

140. Mitotropic EGCg-Functionalised Nanoparticles Efficiently Targets Cancer Cells in a Laminin Receptor-Dependent Manner

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The Investigation of the salient roles of mitochondria dysfunction in the etiology of certain disease conditions has been one of the highlights of medical research in the past century [1]. Mitochondria, being at the nexus of cellular metabolism modulates energy production, and also plays a vital role in apoptosis signaling. A group of mitochondrial-active chemotherapeutic agents has been shown to initiate apoptosis signaling in tumors by their direct impact on mitochondrial function [2]. Also, the characteristic dysfunctional mitochondria of tumors — a consequence of their preference for the rapid generation of ATP through the glycolytic pathway, makes them more susceptible than healthy cells to these agents [3]. Besides the potential selectivity of this approach, mitochondria-targeted therapy has the potential to improve the efficacy of chemotherapeutics, as mitochondria are targets present in all tumors. The objective of this study was to design, characterize and optimize Epigallocatechin gallate (EGCg) reduced gold nanoparticles (AuNPs) for efficient delivery of therapeutics to the mitochondria. Pertinent to this objective is the high affinity of EGCg for the 67 kDa laminin receptor (67LR) that is overly expressed in certain tumors. AuNPs were synthesized by reducing gold (III) chloride with EGCg — a polyphenol from green tea, at ambient temperature and pH 7. The AuNPs were either coated with Poly (Ethylene Glycol) (PEG) (**p-Eg-Au**) or Poly (L-Lysine) -graft- Poly (Ethylene Glycol] copolymers (PLL-g-PEG) (**Pp-Eg-Au**). Both nanoformulations were further decorated with a delocalized Triphenylphosphonium cation (TPP^+), a lipophilic cation that targets the significantly high membrane potential ($\Delta\Psi m$) of cancer mitochondria. Following characterization using transmission electron microscopy, nano-tracking analyzer, FTIR and UV/VIS spectroscopy, we determined the cytotoxic effects and mitochondrial targeting properties of drug-NP conjugates in 67LR negative and positive cancer cell lines, HepG2 and Caco-2 respectively. The drug delivery efficiency of the NPs was demonstrated with the mitochondria-active, but hydrophobic Betulinic acid (BA), a natural pentacyclic triterpenoid. Enhanced cellular uptake, with significant localization to the mitochondria, were recorded for targeted nanoparticles compared to the non-targeted NPs, with uptake levels markedly higher in the 67LR positive cell line. Consistent with these results, significantly high cytotoxic effects of targeted T-Pp-BA-Eg-Au and T-p-BA-Eg-Au compared to the non-targeted constructs and free BA were also recorded. Results from the *ethidium bromide*/acridine orange (EB/*AO*) staining suggests apoptosis as the main cause of cell death. These findings emphasize the innate self-targeting property of Eg-Au, their capacity to modulate drug pharmacodynamics and efficiently deliver drugs to cancer mitochondria.
References ADDIN EN.REFLIST [1] R. Wen, B. Banik, R. K. Pathak, A. Kumar, N. Kolishetti, and S. Dhar, «Nanotechnology inspired tools for mitochondrial dysfunction-related diseases,» *Advanced drug delivery reviews,* vol. 99, pp. 52-69, 2016.[2] J. Neuzil, L.-F. Dong, J. Rohlena, J. Truksa, and S. J. Ralph, "Classification of mitocans, anti-cancer drugs acting on mitochondria," *Mitochondrion,* vol. 13, pp.

199-208, 2013. [3] S. Fulda, L. Galluzzi, and G. Kroemer, "Targeting mitochondria for cancer therapy," *Nature reviews Drug discovery,* vol. 9, pp. 447-464, 2010.

141. PDCD4 Facilitates Apoptosis by Inhibiting of p62 Expression in Human Lung Cancer Cells

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The p62/SQSTM1 (sequestosome 1) protein involved in the regulation of autophagy and apoptosis cell death. Highly expressed of p62 contribute to the development and progression of human cancers. However, the molecular mechanism of p62 can be regulating apoptosis is still unknown. Here, we studied that the effect of PDCD4 on p62 expression in various cancer cells. Our result showed that stably overexpressing PDCD4 suppressed p62 transcriptional activity and promoted apoptosis in human non-small lung adenocarcinoma A549 cells. Additionally, siRNA-mediated p62 downregulation enhanced caspase3 activation and initiate apoptosis in Pdcd4 overexpressed cells, while p62 overexpression inhibited caspase-dependent apoptosis. Moreover, PDCD4 overexpression inhibits p62-dependent target gene expressions as well as Nrf2 nuclear translocation. The knockdown of Nrf2 by siRNA increased apoptosis proteins (caspase3, caspase9, PARP) in Pdcd4 stably overexpressed cells. Taken together, this study demonstrates that PDCD4 induced apoptosis via inhibition of p62 and p62-dependent target gene expressions. These findings offer novel opportunities for p62 targeted interventions to prevent or treat cancer.

142. Long Intergenic Non-Coding RNA 00301 Facilitates Tumor Progression in Non-Small Cell Lung Cancer by Regulation of HIF1α Pathway

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Long non-coding RNAs (lncRNAs) are widely involved in the tumorgenesis and metastasis of multiple tumors. Up to now, the detail molecular mechanism of lncRNA in lung cancer is still largely unknown. Through analyzing the relationship of differential expressed lncRNA and prognosis in lung cancer sample, we found LINC00301 are closed related to prognosis of lung cancer, and the in vitro experiments indicated that LINC00301 inhibited the proliferation, migration and invasion. Combination the prediction of bioinformatics and results of preliminary in vitro experiments, we found LINC00301 can bind to EZH2 to promote H3K27me3 in lung cancer cells. Through the miRDB analysis and RNA-pull down experiment, we found miR-1276 is the potential target of LINC00301, and the expression of HIF1α, the potential target of miR-1276, is closed related with the survival of lung cancer patients. In this project, we plan to combine large numbers of clinical lung cancer tissues, lung cancer cell lines and construction of tumor formation in BALB/c nude mice to explore the roles of LINC00301 in lung cancer, analyze the functional pathways and targets, and identify the detail model of molecular interaction.

143. Assessment of Pathophysiological Role of Free Fatty Acid Metabolic Enzymes under Endoplasmic Reticulum Stress in Lung Cancer

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The endoplasmic reticulum (ER) is the main site of various physiological processes, such as lipid metabolism, protein synthesis and folding, and cellular calcium storage. When cells are stimulated by ER Ca^{2+} depletion, hypoxia, oxidative stress, or nutrient deprivation, the disrupted protein folding induces ER stress which can be resolved via unfolded protein response (UPR) signaling pathways. Multiple types of cancer show increased UPR signaling pathways because ER stress is induced during cancer progression. The cell metabolism is reprogramed to adapt stress condition. Lipid metabolism, including free fatty acid (FFA) metabolism, is also altered under ER stress in cancer. However, the regulation of dozens of enzymes during ER stress is not well-known. In this context, our results revealed that uptake of free fatty acids was suppressed after treatment of ER stress inducer thapsigargin (Tg), an inhibitor of ER Ca^{2+} ATPases, in lung cancer cell lines A549 and CL1-0. It suggests the enzymes related to free fatty acid transport and downstream biogenesis was affected. Our previous study showed overexpression of acyl-coenzyme A thioesterase 11 (ACOT11) and ACOT13 in lung adenocarcinoma. The expression of ACOT13, but not ACOT11, was changed after Tg treatment. To comprehensively explore the unknown FFA metabolic genes, we further examined it in public microarray databases. The several proteins in fatty acid transporter proteins (FATP) family and acyl-CoA synthetase long chain (ACSL) family which involve in FFA transporter, triacylglycerol and phospholipid synthesis, and beta-oxidation was regulated by ER stress in lung cancer. These results provide novel regulatory network in FFA metabolic enzymes in ER stress-mediated lung cancer and therapeutic targets for future human translation.

Cardiovascular and Pulmonary Diseases I

145. Long-Term AAV-9 SERCA2a Gene Therapy Ameliorated Dilated Cardiomyopathy in an Aged Mouse Model of Duchenne Muscular Dystrophy

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Loss of cytoskeletal protein dystrophin in striated muscle leads to Duchenne muscular dystrophy (DMD). A fundamental feature of dystrophic myocytes is the significantly elevated cytosolic calcium level. Increased calcium triggers protein and sarcolemmal degeneration, and eventually myofiber necrosis. Cytosolic calcium recycling is regulated by the sarco/endoplasmic reticulum calcium ATPase (SERCA). SERCA activity is reduced in dystrophic muscle. To test whether SERCA gene transfer can improve calcium recycling and mitigate dystrophic phenotype, we delivered an adeno-associated virus (AAV)-9 SERCA2a vector to 3-m-old mdx mice at the dose of 6E12 viral genome particles/mouse via the tail vein. Whole-body performances were assessed at 8 and 18 months post-treatment using treadmill running and forelimb grip force assays. Serum creatine kinase activity and heart function (ECG and left ventricular catheterization) were examined at 18 months post-treatment. Western blots and immunofluorescence staining confirmed robust SERCA2a expression in heart and skeletal muscle. Forelimb grip strength and treadmill running were significantly improved in treated mice at both time points. AAV-9 SERCA2a treatment also reduced the serum creatine kinase level, a marker of muscle damage. Importantly, SERCA2a treatment significantly improved heart function. Specifically, all ECG parameters were normalized to the wildtype levels. Several measures of systolic function were normalized in the catheter assay. Treatment also significantly improved ejection fraction and the rate of cardiac relaxation. Of notice, treatment significantly improved the end diastolic volume, a prominent feature of the end stage Duchenne dilated cardiomyopathy. Our results suggest that AAV-9 SERCA2a gene therapy is a promising approach to treat DMD (supported by NIH).

146. Wide Spread Adenoviral Vector Delivery to a Cardiac Allograft Utilizing an *Ex Vivo* Perfusion Storage Strategy

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Clinically, cardiac gene therapy has been limited by an inability to achieve wide spread vector delivery to a large volume of the heart. A variety of *in vivo* delivery approaches have all demonstrated therapeutically inadequate transgene expression. Moreover, these approaches pose potential safety concerns associated with extra-cardiac delivery and ectopic transgene expression. Recent developments in the field of normothermic *ex vivo* organ perfusion storage have now created opportunities to overcome these limitations and safety concerns. Our laboratory, using one such device (Organ Care System (OCS) (TransMedics, Inc., Andover MA), sought to investigate the utility of *ex vivo* perfusion as a gene delivery platform to donor hearts prior to transplantation. Components of this delivery platform (proprietary OCS solution, donor blood, and *ex vivo* circuitry tubing and oxygenators) were independently evaluated in a cell-based luciferase assay for their ability to influence efficiency of viral vector transduction. We found that while the OCS solution and the *ex vivo* circuitry did not influence viral vector transduction, the donor blood negatively impacted viral vector transduction (31-fold decrease). We further determined that this is due to serum or plasma fractions which significantly inhibited vector transduction by 535- and 43- fold, respectively. Subsequent gene delivery experiments utilized a CellSaver (Brat 2) Autologous Blood Recovery System to remove undesired plasma or serum components of the donor blood prior to its placement into the circuit. This optimized *ex vivo* perfusion strategy was utilized to deliver $5x10^{14}$ total viral particles of an Adenoviral CMV-luciferase vector to a porcine donor heart prior to heterotopic implantation. We have evaluated the overall levels of expression, protein activity, as well as the biodistribution of the firefly luciferase protein in a series of three transplants. Enzymatic assessment of luciferase activity in tissues (native heart, donor heart, liver etc.) obtained post-operative day five revealed wide-spread and robust luciferase activity in all regions of the transplanted heart (right and left atria, right and left ventricles, coronary arteries) compared to the native heart of the recipient. Importantly, luciferase activity in recipient liver, lung, spleen, or psoas muscle (adjacent to the implanted heart) was within background levels. Similarly, the luciferase protein expression as judged by immunohistochemistry appeared uniform and robust across all areas and levels of the myocardial tissues examined as well as in the coronary arteries. *Ex vivo* heart perfusion opens up a new method of viral vector delivery that can be used to modify donor hearts prior to transplantation to help improve post-cardiac transplant outcomes.*MB and JR contributed equally as first authors in this work

147. Rescue of Genetic Cardiac Arrhythmias by AAV Delivery of the Cardiac Calsequestrin Gene

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Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited cardiac arrhythmia triggered by adrenergic stimulation that can result in sudden death despite current treatment regimens. A recessive inherited form of CPVT is caused by mutations in the *CASQ2* gene which encodes the cardiac calcium binding protein calsequestrin 2 in myocytes. A published mouse model (Circ. Res., v103, p298, 2008) of this disease has been engineered to carry the homozygous disease-causing missense mutation R33Q which closely models the human disease. Correction of the CPVT phenotype in mice with systemic AAV vectors has been described (Circ. Res., v110, p663, 2012). To support translation of this therapeutic strategy towards human application, we designed AT307 to express a codon-optimized version of the human *CASQ2* coding sequence driven by the desmin promoter in an AAV8 vector. We report here the preliminary determination of the minimal efficacious dose (MED) for this product in the R33Q mouse model. R33Q mutant mice received an intravenous (IV) dose $3x10^{12}$ (low), $3x10^{13}$ (mid), or $3x10^{14}$ (high) vg/kg of AT307 and were evaluated for biodistribution and efficacy of reversing the CPVT phenotype. In the mouse hearts, we observed dose-dependent increases in vector copy number, vector derived RNA and protein expression by qPCR, RNA-Seq, Western blotting and immunostaining. Upon immunostaining for calsequestrin, low dose treated mouse hearts exhibited faint cellular staining similar to vehicle-treated R33Q mice, while the mid dose treatment resulted in over 50% of cardiomyocytes strongly staining, while nearly all cells were similarly positive at the high dose. To assess phenotypic correction, electrocardiograms (ECG) were performed on the mutant mice after epinephrine stimulation and scored in a blinded fashion for the presence or absence of arrhythmias characteristic of CPVT. Four weeks after AT307 dosing, 14 of 19 mice treated with vehicle exhibited arrhythmias as did 19 of 20 mice receiving the low dose. In contrast, 0 of 23 mid dose mice and 3 of 22 high dose mice exhibited arrhythmia at that time. These data further support the strategy of using systemic dosing to achieve correction of inherited cardiac arrhythmias, and establish a preliminary MED of $3x10^{13}$ vg/kg for the treatment of CASQ2-CPVT with AT307.

148. Functionally Enhanced Cystic Fibrosis Transmembrane Conductance Regulator Expression by dCas9-VPR and BGas siRNA Nanoparticles

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Cystic fibrosis (CF) is a hereditary disease caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which results in reduced functional CFTR protein and ultimately reduced transport of chloride, bicarbonate, sodium and water in epithelial tissue. Strategies to both bolster CFTR expression as well as facilitate functional protein reaching the cell surface could result in a therapeutic approach to enhancing long-term survivorship. To determine the range of CFTR to transcriptional activation, as a putative CFTR specific therapeutic, we contrasted siRNAs nanoparticles, targeted to activate CFTR by inhibition of the CFTR regulatory antisense lncRNA, BGAS, with nuclease dead Cas9 (dCas9) fused to transcriptional activator domains. We show here a guide RNA, (G-3) directed defective CRISPR fused to the VPR activator (dCas-VPR) targets the CFTR promoter and results in a marked increase in CFTR mRNA and functional protein expression. We also find that siBGas4 can functionally activate CFTR when delivered with the Tat cell penetrating peptide (GRKKRRQRRR) in a nanoparticle conjugate. We find from a screen that one candidate, 9T containing GRKKRRQRRR in a non-covalent complex with siRNA, demonstrated a dose dependent activation of CFTR. Collectively, these observations suggest that targeted small RNA and RNA/dCas-VPR (CRISPR) approaches can target and activate CFTR. Notably, these two approaches can be used either together or individually to enhance CFTR expression. Funding: NIDDK R01 DK104681-01 to KVM.

149. Vascular Targeting of mRNA Provides Orders of Magnitude Elevation of Delivery and Transgene Expression Selectivley in the Lungs

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Recent progress in therapeutic mRNA-based nanomedicine has created excitement amongst biopharmaceutical and clinical researchers for its use as a practical and efficient therapeutic platform. Numerous acute cardiopulmonary pathological conditions still lack adequate therapies due to limited efficacy of current small molecules and protein therapeutics. A pressing need exists in the field for fundamental components, such as mRNA that have strong, transient, safe and controllable delivery. Here, we demonstrate active targeting of reporter mRNA containing lipid nanoparticles (LNPs) decorated with affinity moieties against vascular endothelium. *In vivo* radiotracing of targeted LNP carriers was used for accurate pharmacokinetic and biodistribution analysis. Moreover, functional activity of the mRNA cargo was evaluated using luciferase assay and bioluminescence imaging. Selective pulmonary uptake and luciferase transfection was observed in mice treated with LNPs targeted to Platelet-Endothelial Cell Adhesion Molecule-1 (PECAM) and Intercellular Adhesion Molecule-1 (ICAM-1). Unlike hepatic delivery of unconjugated LNP-mRNA, vascular-targeted LNP-mRNA bypasses serum components such as apolipoprotein-E. To further expand the targeting platform to pathologic states, we investigated the efficacy of the system in a LPS inflammation mouse model, in which, ICAM-1-targeted LNPs effectively localized in the inflamed lungs after IV injection.

150. Exosomes Based Therapy for the Treatment of a Mouse Model of Lung Injury Using Extracellular Vesicles Derived from Umbilical Cord-Derived Mesenchymal Stem Cells

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BACKGROUND: Cell therapy using stem cells has produced therapeutic benefits in animal models of idiopathic pulmonary fibrosis (IPF). Secretory mediators are proposed as one mechanism for the stem cell therapeutic benefits observed, such as modulating the inflammatory and fibrotic pathways. Recently, exosomes have been shown to have immunomodulatory properties similar to that of mesenchymal stem cells (MSCs). In our present report, we investigated the anti-inflammatory, anti-fibrotic and pro-regenerative properties of exosomes derived from umbilical cord-derived mesenchymal stem cells (UC-MSCs) in a mouse model of lung injury. **METHODS:** Exosomes were purified after UC-MSCs were cultured in hypoxia by differential centrifugation and identified by transmission electron microscopeexamined using nanoparticle tracking analyses. Lung injury in mice was induced by administrating 0.04 units of bleomycin intranasally. Animals were treated at day 1 (early) and day 7 (late). The impact of the exosomes on the immuneresponse, histological outcomes, and bronchioalveolar cell response were assessed in vivo. **RESULTS:** Exosomes derived from UC-MSCs are enriched in miRNAs (P13K-Akt, Ras, Hippo, TGF-B), these miRNAs have been shown to be involved in MAPK signaling and apoptotic pathways, in addition to proteins associated with PDGF, and FGF signaling. Intranasal delivery of the UC-MSCs exosomes at 15ug at Day 1 following bleomycin challenge significantly reduced lung inflammation. Exosome treated mice demonstrated a twofold reduction in collagen ($p \leq 0.01$) and a reduction in alpha-SMA(+) cells. **CONCLUSIONS:** In summary this study demonstrates that exosomes derived from UC-MSCs can deliver a strong therapeutic effect of anti-fibrotic, immunomodulatory and regenerative properties in a mouse lung injury model. This is the first study describing the intranasal delivery of exosomes derived from UC-MSCs. Current treatments using nintedanib and pirfenidone targets the fibrotic phase and has limited effects of treating lung disease, these results indicate that an alternative therapeutic exosome based treatment is possible.

151. Evaluation of miRNA-181-5p Forced Expression Umbilical-Cord Derived Mesenchymal Stem Cell Exosomes in a Acute Myocardial Ischemic Injury Model

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BACKGROUND Recent studies have indicated that exosomes play an important role in providing the therapeutic benefits and paracrine interaction been cells and tissues. The aim of this study is to investigate whether exosomes derived from umbilical cord-derived mesenchymal stem cells (UC-MSCs) enriched with microRNA miRNA-181-5p have a more protective effect on acute myocardial infarction (AMI). **METHODS:** Exosomes were purified after UC-MSCs were cultured in hypoxia by differential centrifugation and identified by transmission electron microscope examined using nanoparticle tracking analyses. A rat model of myocardial infarction and in vitro model of hypoxia-induced myocardial cell injury were established in order to determine if there were any therapeutic benefits of exosomes from miRNA-181-5p overexpressing UC-MSCs. **RESULTS:** The in vitro results demonstrated that exosomes derived from miRNA-181-5p-overexpressing UC-MSCs decreased myocardial cell injury by reducing the inflammatory response during hypoxia induction. The miRNA-181-5p-enriched exosomes also decreased the expression of fibrosis-related proteins of under hypoxic conditions. In vivo studies confirmed that exosomes derived from UC-MSCs significantly decreased the myocardial injury area of infarction, especially after miRNA-181-5p enriched exosome treatment. Cardiac fibrosis and inflammatory cytokine expression were also decreased after treatment with miRNA-181-5p-enriched exosomes. H&E-stained cross-sections of the myocardium indicated that the injection of miRNA-181-5p enriched exosomes significantly improved the MI area-to-LV ratio when compared across other control treatment groups. Both IL-1β and IL-6 mRNA expression decreased in the infarct in the miRNA-181-5p enriched exosomes treated animals. **CONCLUSIONS:** The results suggested that the expression of miRNA-181-5p-enhanced UC-MSCs derived exosomes attenuated myocardial damage by protecting myocardial cells from apoptosis, inflammation, fibrosis, and increased angiogenesis compared to controls in vitro and in vivo. These results, provide an effective anti-fibrotic function of engineered UC-MSCs cultured under hypoxia and may be a novel cell-free therapeutic approach for treating ischemic diseases.

152. Expression Profile of Optimised SIV Vector in the Mouse Lung at Early Timepoints Post-Delivery

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The UK Cystic Fibrosis Gene Therapy Consortium are developing a lentiviral (LV) gene transfer vector to treat Cystic Fibrosis lung disease. Our preferred vector (rSIV.F/HN) is based on recombinant simian immunodeficiency virus (SIV), pseudotyped with the F/HN proteins of Sendai virus to promote efficient airway cell uptake (Mitomo et al., 2010, PMID 20332767). Crucial to achieving long term clinical progress, is understanding the expression profile kinetics of lentiviral vectors in-vivo and the design of assays to quantify successful transduction in tissue samples. The nature of LV vectors makes this process difficult as they contain sense strand mRNA potentially capable of expressing in target cells, in the absence of, or prior to, integration of the LV genome. Ultimately this could have consequences for long-term activity by inducing an antitransgene immune response, yet the majority of in-vivo studies with LV vectors do not consider this possibility. We therefore sought

to determine the levels of LV activity in-vivo at very early time-points. Female BALB/c mice (n=10) were dosed intransally with 1e7 Transducing Units per ml (TU/ml) of an LV vector expressing luciferase under the control of a CMV promoter. Bio-luminescent imaging of live mice was used to determine Luciferase activity at 0h, 3h, 6h, 12h, 24h, 2d, 3d, 7d, 14d & 28d post dosing. In parallel, separate mice (n=6 per timepoint) received the same dose of vector. The lungs and nasal tissue were harvested at 0h, 6h, 12h, 24h, 2d, 7d and 14d post-dosing for mRNA and DNA analysis by rtPCR. The mRNA data at early timepoints exhibited peak levels in the first 6 hours post-delivery, confirming our hypothesis that measurement of mRNA at these early time-points is simply a measure of vector delivery. Subsequently, the mRNA levels fell sharply (over 800 fold) by day 7 post-delivery and were then stable to day 14. The bioluminescent imaging data for luciferase showed a different profile. During the first 24 hours after delivery, luciferase activity was around background levels, with activity starting to rise slightly at day 2 and day 3 and peaking at day 7 post-delivery. Activity then fell slightly at day 14 and day 28. Interestingly, vector DNA was largely undetectable by rtPCR prior to day 7. This suggests that significant transgene activity following LV delivery is only detected following reverse transcription and integration of the vector DNA. These data indicate that at early time-points post-delivery mRNA levels are not an accurate marker for expression from LV, but is simply a measure of vector delivery. Interestingly, despite the initially very high levels of vector RNA detected in the lungs and nasal tissues of mice, this was not associated with measurable levels of luciferase (protein) activity. Indeed, protein activity from the LV was not observed until measureable levels of vector DNA were also present. Encouragingly, these data demonstrate that despite the highly detectable delivery of sense strand mRNA, no burst of protein production is observed at early timepoints, which might be detrimental to long-term LV activity. These findings also help inform the timing of assays designed to quantify transgene expression post-delivery in toxicology and clinical studies.

153. Proresolutive Effects of GM-CSF and M-CSF Gene Therapies in the Limb Ischemia

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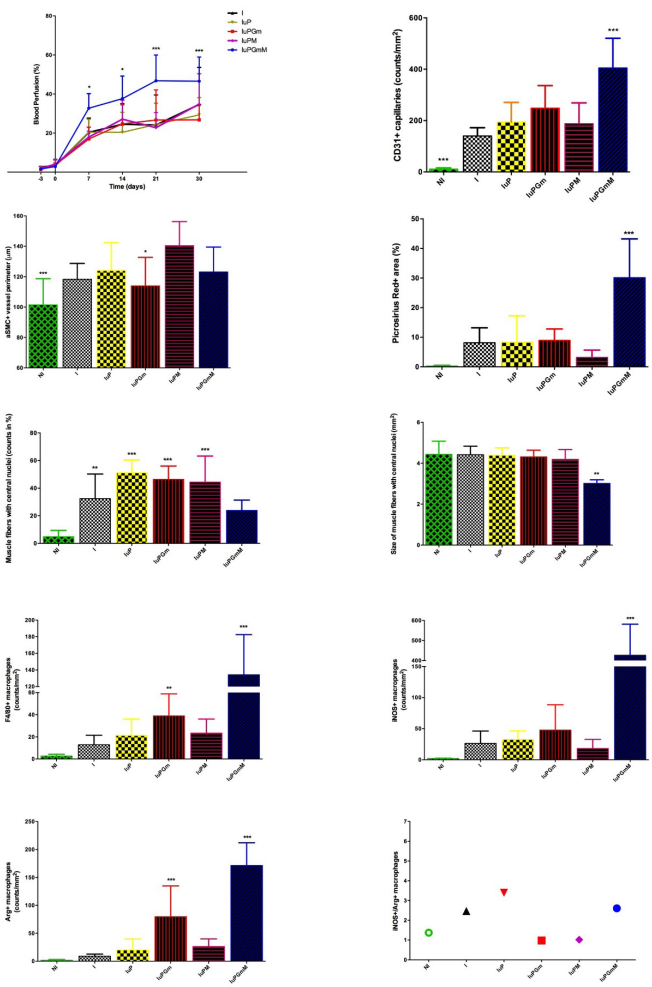
Background: Peripheral arterial disease is characterized by the narrowing or occlusion of the blood arteries. The formation and remodeling of vessels to ameliorate ischemic tissues depend mainly on the type of macrophages present at the site. GM-CSF and M-CSF are colony-stimulating factors that act in synchrony on hematopoiesis. Whereas GM-CSF has a broader action, which includes the maintenance and mobilization of hematopoietic stem cells and formation of granulocytes, monocytes and macrophages, M-CSF is more specific for differentiation and proliferation of monocytes and macrophages. Based on these information, we proposed that the administration of GM-CSF and M-CSF 2 days later can form more proresolutive macrophages to treat limb ischemia. Methods: Limb ischemia was induced surgically by removal of femoral artery of Balb/c male mice of 10 to 12-week-old mice. After three days, rectus femoris muscles were electroporated with 100 μg uP-mGMCSF in PBS and two days later with 100 μg uP-mMCSF. Weekly, blood flux was assessed by laser Doppler image system and limb necrosis by visual scoring; Complete blood count (CBC) was also performed using blood samples collected weekly. Thirty days after the last transfection, mice were euthanized, and the gastrocnemius muscle mass and force were measured. These muscles were analyzed by histology and immunohistochemistry (IHC) later. In this study, the following groups were included: I (ischemic mice), IuP (I + uP), IuPGm (I + GM-CSF), IuPM (I + M-CSF), IuPGmM (I + GM-CSF and M-CSF) and NI (non-ischemic mice). uP is the gene expression vector. For statistical analyses, ANOVA was used followed by Tukey post hoc test. Results and Discussion: Ischemia caused necrosis in the fingers and toes of only some mice, and there were no significant statistic variations among all groups. CBC also showed no significant variation among groups. A few days after ischemic surgeries almost no blood flux was observed in all groups (Figure 1). IuPGmM group recovered the blood flux faster than other groups and reached 46.6% at the day 30 in comparison to the contralateral limbs. GM-CSF and M-CSF treated groups did not vary in comparison to the control groups I and IuP. However, the recovery of muscle force/mass (Newton/gram) was better in IuPM group (2.3) than IuPGm (1.5) or IuPGmM (1.1), which in turn were much better than the control group IuP (0.4). The NI group showed 3.5. Histology of the skeletal muscles after 30 days showed IuPGmM group with more vessel density than IuPGm or IuPM, but this group also produced more fibrosis in the skeletal muscle. Consequently, less myofibers were observed in IuPGmM group. Macrophages subpopulations analyzed by IHC shows higher macrophage populations in the IuPGm group than the IuPM, but the treatment by both CSFs resulted in an even greater presence of all macrophages. The ratios of iNOS+/Arg+ macrophages of IuPM and IuPGm were 1 and of IuPGmM was 2.6. Collectively, our study shows that double gene therapy with GM-CSF and M-CSF produces more proinflammatory macrophages in loco, resulting faster angiogenesis and blood flux recovery than single gene therapies. However, the fast circulation recovery did not reflect in the fast recovery of functionality. Transfection of each gene separately favored the formation of more proresolutive macrophages and improved physiological recovery. Financial supports: FAPESP and CNPq



154. Airway Gene-Addition Therapy for Cystic Fibrosis: the VSV-G Pseudotype Produces Higher Transduction Levels Than HA

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Introduction Lentiviral (LV) vectors are a promising option for treating Cystic Fibrosis (CF) airway disease by delivering a functional copy of the CFTR gene into airway epithelial cells, but envelope choice is important to ensure that the desired cells are efficiently targeted. The vesicular stomatitis type-G (VSV-G) and the Hemagglutinin (HA) pseudotypes target airway receptors on the basolateral and apical surfaces, respectively. The aim of the project was to use reporter genes to determine which pseudotype is more effective, and whether brief perturbation of airway surface tight-junction integrity with lysophosphatidylcholine (LPC) prior to vector delivery increases transduction efficiency. **Methods** The lungs of intubated normal C57Bl/6 female mice received 10 µl of PBS (control; n=8-12) or LPC (n=8-12), followed one hour later by two 15 µl aliquots of a VSV-G (n=8-12) or HA (n=8-12) pseudotyped LV vector containing either the *LacZ* or *Luc* reporter genes. One week post LV vector instillation, mice that received the *LacZ* reporter gene were humanely killed and LacZ transduction was assessed *en face*. For the mice that received the *Luc* reporter gene, bioluminescence imaging (BLI; Xenogen, IVIS) was performed at 1 week, and then monthly for up to eight months after LV vector instillation to assess Luc expression levels. **Results** *En face* assessment of mouse lungs showed that LPC conditioning resulted in stronger initial LacZ transduction levels than PBS, independent of which pseudotype was used. In the VSV-G treated mice, LacZ transduction was typically more pronounced in the trachea and upper bronchioles, regardless of whether the conditioning was with PBS (control) or LPC. In the HA treated mice, higher levels of transduction were observed in the trachea and bronchioles after LPC conditioning, compared to PBS. Lung luminescence was detected by BLI at all imaging time points, regardless of the conditioning or pseudotype. At one week, the VSV-G group had significantly higher expression levels than the HA group, regardless of whether LPC was used ($p>0.0001$, two-way ANOVA, Tukey's). Longer term (1 to 8 months) there was no quantifiable difference in luminescence between groups pre-treated with PBS or LPC, or the VSV-G or HA pseudotypes. **Conclusion** At one week the VSV-G pseudotype was more effective at transducing airway cells than the HA pseudotype. The long-term Luc results suggest that conditioning the airways with LPC prior to LV vector delivery does not increase the total lung transduction levels with either pseudotype. Monthly BLI assessment is being continued to observe total lung gene expression levels over time. The location and type of cells transduced in conducting and gas exchange airways will be assessed in the LacZ group. **Acknowledgements** Channel 7 Children's Research Foundation, Cure4CF Foundation, NHMRC. Thanks to John Olsen (UNC) for access to and discussions about the HA pseudotype.

155. Augmentation of Angiogenic Activity of Platelet Rich Plasma by Colostrum Derived Immunoglobulin

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Background: Platelet rich plasma (PRP) is known to containing multiple growth factors and previously has been demonstrated to induce angiogenesis in various settings. The ease of generation, ability to manufacture autologous preparations, and established safety record supports possible use in stimulation of angiogenesis. Bovine colostrum has been reported to possess several growth factors increasing IGF and various antibodies. **Materials and Methods:** In vitro assessment of angiogenesis was performed by quantification of proliferation of human umbilical vein endothelial cell (HUVEC) assay in response to PRP, colostrum, and the combination. In vivo administration of PRP alone, colostrum alone, or the combination was assessed in a murine hindlimb ischemia model. Limb viability was assessed up to date 30. **Results:** Synergy of HUVEC proliferation was observed by combination of PRP and colostrum. Fractionation

studies demonstrated proliferative component was associated with immunoglobulin content in a dose dependent manner. Paradoxically, human intravenous immunoglobulin (IVIG) suppressed HUVEC proliferation. Administration of colostrum, or colostrum derived immunoglobulin synergized with PRP, but IVIG administration inhibited PRP ability to enhance limb survival. **Conclusions:** These are the first studies to our knowledge that antibodies derived from colostrum possess angiogenesis stimulating activity. Given the established safety profile of PRP and colostrum, further studies are warranted to determine whether this combination may be useful in treatment of limb ischemia.

156. Gene Electrotransfer and Myocardial Repair in a Rat Model

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A leading cause of heart failure and a major public health concern is atherosclerosis of the coronary vessels, causing myocardial ischemia and infarction. Here we examine a protective/angiogenic and potentially regenerative/cardiomyogenic gene therapy approach to reducing myocardial infarct size in a rat model. Gene electrotransfer (GET) was assessed as a delivery method of plasmid encoding vascular endothelial factor B (VEGF-B) to ischemic myocardium in a rat model. Electrotransfer parameters, delivery electrode design, timing and duration of pulses relative to the electrocardiogram and ischemic state were considered for efficient gene delivery and gene expression modulation. We used a rat animal model for gene delivery to ischemic and non-ischemic left ventricular myocardium. Gene expression of reporter genes was evaluated histologically for expression distribution as well as quantitatively via bioluminescence imaging. Gene delivery of an effector gene encoding VEGF-B was performed in a myocardial infarction model. A control group of animals received saline injection to the ischemic myocardium without electrotransfer. The animals were followed from 2 up to 56 days post treatment. Hearts were collected at various time points and were evaluated histologically for infarct size, cell proliferation and cardiomyogenesis via Masson's trichrome staining, Ki67 and BrdU immunoreactivity respectively. Enhanced levels and transfection efficiency were achieved with GET mediated plasmid DNA delivery to the beating ischemic and non-ischemic myocardium over controls. Gene expression was distributed in the myocardium and the pericardium. Infarct size was significantly reduced in pVEGF-B gene delivery groups. New cardiomyocytes were observed suggesting a regenerative role for VEGF-B in addition to the well-documented protective (angiogenic) role leading to infarct size reduction. Therefore, GET mediated delivery of pVEGF-B to ventricular myocardium in a small animal model can be accomplished safely with favorable gene expression kinetics and distribution. Additionally, GET mediated delivery of pVEGF-B may enhance myocardial repair post infarction or ischemia onset via the process of cardiomygenesis.

Cell Therapies I

157. Neural Stem Cell-Delivered Chimeric Pox Virotherapy for Ovarian Cancer

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Ovarian cancer is the most lethal gynecologic malignancy, afflicting approximately 22,000 women per year in the U.S. Once ovarian cancer has metastasized to the abdominal cavity (stage III), patients have only a 34% 5-year survival rate following standard treatment with surgical debulking and combination chemotherapy. Use of intraperitoneally (IP) delivered combination chemotherapy regimens has improved outcomes; however, these regimens frequently have complications and serious toxic side effects that prevent most patients from completing full treatment cycles. Additionally, most ovarian cancer patients eventually develop chemoresistance, leading to cancer progression and death. Replication-competent oncolytic virotherapy offers a new, highly promising approach for treating ovarian cancer. Once seeded into the tumor, the oncolytic virus can selectively replicate in tumor cells (but not in normal tissue) to destroy tumor cells in situ via direct lysis. Importantly, oncolytic viruses can induce cancer cell death irrespective of radio- or chemoresistance and can also stimulate immune system recognition of cancer cells by exposing tumor antigens upon lysis. Clinical efficacy of this approach has been limited by rapid viral inactivation by the immune system, poor viral penetration of tumors, and an inability of the virus to effectively reach invasive metastatic foci separated by normal tissue. We now present a novel conditionally replication-competent chimeric orthopoxvirus (CF33) selected specifically for ovarian cancer cell infection, following screening of over 100 chimeric poxviruses. The CF33 virus was engineered to be tumor specific by deleting the virus's thymidine kinase (TK) gene. Tumor tissues have abnormally high TK expression that complements this gene deletion, permitting viral replication, whereas normal tissue does not. We have modified a tumor-tropic, HLA II-negative neural stem cell line (HB1.F3.CD21 NSCs; demonstrating clinical safety in high-grade glioma patients) that efficiently delivers CF33 to ovarian cancer foci in preclinical models. We hypothesize that NSCs will provide protection from immune-mediated clearance and neutralization to achieve effective viral distribution to peritoneal ovarian tumors. Co-culture *in vitro* experiments of NSC-delivered CF33 in human OVCAR8 and murine ID8 ovarian cancer cells showed robust infection of >95% of tumor cells in 6 days, even at a low ratio of 1 NSC: 1000 tumor cells. *In vivo* data show selective distribution of NSC-delivered CF33 to human OVCAR8 and ID8 peritoneal metastases in immunodeficient and immunocompetent mice, respectively. Furthermore, IP-delivered CF33-NSCs showed increased viral distribution to tumor sites (assessed by BLI, IHC, and qPCR) compared to a matched viral load of free CF33 when multiple treatment rounds were given in the immunocompetent model. Long-term efficacy studies are in progress to establish the clearance kinetics, immune response, viral distribution, and efficacy of NSC-delivered vs. free CF33. Our long-term goal is to demonstrate efficacy and safety of CF33-NSCs for selective tumor killing in patients suffering from stage III ovarian cancer.

158. Efficient Genome Editing of Immune Cells Using Microfluidic Delivery as a Novel Approach to Cell Therapy

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The translational potential of cell-based therapies is often limited by complications related to effectively engineering and manufacturing cell therapies. For example, delivery of CRISPR-CAS9 ribonucleoproteins (RNPs) to sensitive cell types (primary cells) using current technology can significantly impact viability and function. Our microfluidic approach to delivery, CellSqueeze® (SQZ), which mechanically deforms cell membranes through constricting channels, results in diffusion of material from the surrounding buffer directly into the cytosol. By eliminating the need for electrical fields or exogenous materials such as viral vectors, SQZ minimizes the potential for cell toxicity and off-target effects observed with conventional delivery methods. These advantages make SQZ a potential platform for enabling challenging cell therapies including the use of CRISPR to engineer cell function. Here, we demonstrate that SQZ-mediated engineering of immune cells results in the desired phenotype with minimal effect on viability, gene expression, and cell function. To determine the effect of membrane deformation on gene expression compared to electroporation (EP), treated cells were compared to unmanipulated control cells using microarray analysis. Differential gene expression for both delivery methods was assessed by t test on the coefficient of a linear mixed-effects model that treated delivery method as a fixed effect and donor as a random effect. EP produced substantially more gene expression changes than SQZ, particularly for activation markers. To determine if *in vivo* functionality was also impacted, CD3+ OT-1 T cells were isolated from CD45.2 mice, treated with SQZ or EP, and injected into CD45.1 mice. Mice were challenged with SIINFEKL peptide subcutaneously three hours post-injection; splenocytes from lymph nodes and spleens were collected four days post-injection, re-challenged with SIINFEKL, and stained intracellularly. The percentage of IFNγ+ cells and mean fluorescence intensity (MFI) of IFNγ+ cells in the EP arm (55%, 5,848 AU, respectively) were significantly reduced compared to no-treatment control (74%, $p<0.001$; 9,488 AU, $p=0.03$) and squeezed cells (72%, $p<0.001$; 8,753 AU, $p=0.01$). In contrast, there was no significant difference between control and SQZ for either measurement of IFNγ secretion, suggesting that squeezed T cells maintain potent effector function *in vivo*. Subsequently, we designed a series of experiments to manipulate gene expression with the CRISPR system using SQZ to deliver CAS9 RNPs, recombinant CAS9 protein complexed with a single-guide RNA designed to edit a checkpoint protein (CP). Delivery of RNPs *via* SQZ to T cells resulted in >53% reduction in CP surface protein levels by FACS analysis, with genetic knock-down confirmed at >49% by T7 assay. To test the functionality of SQZ-mediated CP-knockdown, we injected edited OT-1 mouse T cells into CD45.1 mice (SQZ-RNP group), then subcutaneously injected E.G7 Ova tumor cells 7 days later. Seventeen days post-tumor injection, the SQZ-RNP group had dramatically smaller tumors (60 mm^3) compared to SQZ alone (524 mm^3) and naïve (880 mm^3). Tumor-infiltrating lymphocyte (TIL) analysis resulted in more CD8+ (45% *vs* 19%) and fewer CP+ (39% *vs* 96%) T cells in SQZ-RNP arm compared to SQZ only controls, suggesting the decreased tumor size was a result of more robust response and clearance by SQZ-mediated CP knockdown cells. These data suggest that SQZ is a viable delivery method for genetic engineering of primary T cells with few off-target effects on baseline gene expression and function. Indeed, the ability to deliver structurally diverse materials to difficult-to-transfect primary cells indicate that this method could potentially enable many novel clinical applications.

159. Engineering Red Blood Cell-Based Biosensors for Physiological Monitoring

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Cell-based therapies have a wide range of applications ranging from cancer immunotherapy to regenerative medicine. An emerging frontier of this field is the development of engineered red blood cells (eRBCs) for therapeutic and diagnostic applications. RBCs are an attractive platform because they have exceptionally long circulation times (120 days), lack DNA, and can be loaded with drugs, proteins, or other cargo. Recent technological advances have enabled the large-scale production of RBCs from precursor cells, which may potentially be harnessed to generate off-the shelf eRBC-based products to meet medical needs. The specific goal of this project is to generate eRBC-based technologies enabling non-invasive monitoring for pathogen exposure (e.g., in the context of first responders) and for other "actionable" physiological analytes (e.g., markers of acute inflammation). As a first step towards enabling RBCs to act as sensors, we designed and evaluated a novel biosensor strategy that is suitable for achieving biosensing in eRBCs, which lack DNA and thus require a readout other than gene expression. Towards this end, we engineered a cell-surface receptor protein in which ligand binding induces receptor dimerization, which then facilitates reconstitution of an intracellular split reporter protein. Importantly, our strategy involves modification of RBC-resident proteins, since retention of membrane proteins during RBC maturation is a tightly regulated and incompletely understood process. We comparatively evaluated a range of biosensor architectures, enabling us to identify biosensor designs and design features that successfully conferred significant ligand-induced generation of fluorescent output. We then carried forward the most promising architectures for testing in G1E cells—a murine erythroid cell line—to verify biosensor expression and functionality in a red blood cell model. This study establishes a foundation for developing eRBC biosensors that could ultimately address an unmet need for non-invasive monitoring of physiological signals for a range of diagnostic applications.

160. Comet: A Novel Transcription Toolbox for Engineering Cellular Therapies

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Engineering mammalian cells to serve as therapeutic or diagnostic devices relies upon technology for controlling gene expression. In many applications, signals received by receptors or other biosensors are ultimately transduced into a gene expression event, such as the production of a therapeutic protein or diagnostic readout, through a pathway comprising one or more transcription factors. Customized gene expression programs, including those developed using the approaches of mammalian synthetic biology, are currently limited to using either native transcription factors, which are subject to cross-regulation and interference with native cellular functions, or to using a small set of engineered transcriptional regulators, such as tTA and Gal4. To realize the goal of building sophisticated cellular programs, a larger set of orthogonal, well-characterized transcriptional regulators is needed. Towards this goal, we have developed and characterized the Composable Mammalian Elements of Transcription (COMET)—an ensemble of transcription factors and cognate promoters that enable the design and tuning of mammalian genetic circuits to an extent not previously possible. COMET currently comprises 53 activating and 38 inhibitory zinc-finger-based regulators, built using modular domains, and 84 cognate promoters; this system also enables the rapid construction of additional parts as needed for specific applications. These parts were systematically characterized to generate a quantitative map of gene regulation properties. We demonstrated the utility of COMET by modulating gene expression over three orders of magnitude, introducing chemically inducible control, and by implementing single-layer design-driven, sophisticated Boolean logic. Furthermore, we developed a mathematical model that accurately explains these behaviors and can be applied to guide the design of new circuits. Altogether, COMET is a powerful tool that enables bioengineers to rapidly design and implement custom genetic programs in mammalian cells.

161. Endogenous TCR Replacement in Human T Cells with Non-Viral Genome Targeting

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Several decades of work have aimed to genetically reprogram T cells for therapeutic purposes, but as human T cells are resistant to most standard methods of large DNA insertion these approaches have relied on recombinant viral vectors, which do not target transgenes to specific genomic sites. In addition, the need for viral vectors has slowed down research and clinical use as their manufacturing and testing is lengthy and expensive. Genome editing brought the promise of specific and efficient insertion of large transgenes into target cells through homology-directed repair (HDR), but to date in human T cells this still requires viral transduction. We have developed a non-viral, CRISPR-Cas9 genome targeting system that permits the rapid, efficient, and scalable insertion of individual or multiplexed large (>1 kilobase) DNA sequences at specific sites in the genomes of primary human T cells while preserving cell viability and function. We applied non-viral genome targeting to replace the endogenous T cell receptor (TCR) locus with a new TCR specificity. Replacement of the endogenous TCR with a new specificity for the NYESO tumor antigen resulted in engineered T cells which specifically recognized the tumor antigen, with concomitant cytokine release and tumor cell killing. Non-virally engineered T cells with a user defined TCR specificity could be produced at clinical scales of hundreds of millions of cells. Taken together, we provide preclinical evidence that non-viral genome targeting will enable rapid and flexible experimental manipulation and therapeutic engineering of primary human immune cells.

162. A Short Investigation of Mixed Haematopoietic Chimerism: An NHP Model of Intrauterine Haematopoietic Stem Cell Transplantation (IUHSCT)

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Introduction Intrauterine hematopoietic stem cell transplantation (IUHSCT), a non-myeloablative approach for prenatal treatment of a variety of congenital hematological and immunological disorders, may serve as an alternative option to mainstream treatment of congenital haemoglobinopathies, with a small cell dose given to the fetus during the period of pre-immunity that may effect a greater engraftment and minimize the risk of graft versus host disease (GVHD) at the same time. We have developed a non-human primate (NHP) model of IUHSCT by injecting maternal bone marrow-derived mononuclear cells (MNCs) into the fetus to determine donor cell engraftment and the feasibility of this approach. Methods Maternal MNCs were isolated and labelled with carboxyfluorescein succinimidyl ester (CFSE) prior to in utero injection into fetus via umbilical cord fetoscopically or transabdominal intra-cardiac puncture under ultrasound guidance, at 0.46-0.74 gestational age (71-144 days). Fetuses (n=10) received 18.9-170 x 10^6 MNCs (median dosage 49.8 x 10^6) and were sacrificed at 24-48 hours post-injection. Targeted organs were harvested for analysis through stereoscopy, flow-assisted cytometry (FACS) and qPCR of MHC polymorphisms. Fetal weights ranged from 36-160g. Results Stereoscopy results revealed detectable levels of CFSE-positive MNCs in fetal liver, spleen, heart, lung and placenta, which were in accordance to FACS results. Low levels of maternal MNCs were found in haematopoietic tissues like fetal liver, spleen and heart (2-8%). In contrast, qPCR results indicated that maternal MNCs were mostly found in placenta (6.9%) and present at relatively low levels in other organs (<0.01%). Conclusions & Future Work It is technically challenging to deliver donor cells via fetoscopy as compared to ultrasound-guided intrauterine injection. Although maternal cell chimerism may be low in some organs, there is hope that the small amount of donor engraftment may help to ameliorate or arrest disease

pathology until further interventions can be carried out post-natally. Moving forward, we will be exploring the ability of early-gestation high dose IUHSCT at 0.4G to achieve stable significant engraftment and generate donor specific tolerance (DST) in our NHP model.

163. Development of a Switchable CAR-T Cell Platform for Clinical Use

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Chimeric antigen receptor (CAR) T cell therapy has recently emerged as a powerful tool against malignancies such as refractory acute lymphoblastic lymphoma (ALL) and diffuse large B cell lymphoma. However, the inability to control the activation of CAR-T cells post-infusion poses safety risks and leads to adverse effects, including cerebral edema and cytokine release syndrome. Herein, we describe a humanized switchable CAR-T (sCAR-T) cell system, which consists of a third-generation CAR with an scFV domain specific to a peptide neo-epitope (PNE), and an antibody-based "switch" with an engrafted PNE. Because sCAR-T cells are activated only when the switch is present, the sCAR-T cell response against malignant cells can be fine-tuned through the switch dosing regimen to minimize adverse effects. In addition, the short half-life of the switch provides the ability to control the duration of sCAR-T cell activation. Finally, one or more tumor antigens can be targeted at the same time or sequentially, providing the ability to treat heterogeneous malignancies. The switch can be engineered to target any tumor antigen, and thus a uniform streamlined sCAR-T manufacturing process can be used to generate therapies against multiple types of cancer. We are currently developing the manufacturing process to produce a switchable CAR-T cell system that will allow treatment of a wide range of malignant diseases in a time- and cost-efficient manner.

164. Delivery of Wnt-11-Expressing MDSCs with FGF2-Loaded Coacervate to Improve Cardiac Repair

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Myocardial infarction (MI) (i.e., heart attack) is the irreversible death (necrosis) of heart muscle which is secondary to prolonged lack of oxygen supply (ischemia). Approximately 1.5 million cases of MI occur annually in the United States. Cardiac tissue regeneration with the application of stem cells may be an effective therapeutic option. Cellular cardiomyoplasty (CCM), which involves the transplantation of exogenous cells into the heart, is a promising approach to repair injured myocardium and improve cardiac function. In recent years, our group has demonstrated that stem cells engrafted with injectable biomimetic coacervate show great potential to repair necrotic myocardium. We have isolated a population of muscle-derived stem cells (MDSCs) from the skeletal muscle of mice and humans which, when compared with myoblasts, display a significantly improved capacity for cardiac regeneration in a mouse model of acute myocardial infarction (AMI). Transplanted MDSCs survive significantly better than skeletal myoblasts due to their high expression of cellular antioxidants, which increases resistance to stress, and a paracrine effect which reduces myocardial fibrosis, promotes angiogenesis, and ameliorates left ventricular (LV) remodeling. However, several hurdles may still limit the cardiac regenerative potential of MDSCs, including poor approaches for delivery of the cells (e.g., direct intramyocardial injection in PBS) that lead to limited cell retention and survival, as well as the low cardiomyogenic potential of MDSCs. We report, here, the use of fibroblast growth factor 2 (FGF2)-coacervate as a novel delivery vehicle for MDSCs, which represents a new area of research and not only promotes cell retention, survival, and cardiac regenerative potential of MDSCs (with or without Wnt-11 transduction), but also synergistically enhances angiogenesis through the release of FGF2. We show that coacervate loaded with FGF2 is capable of enhancing cardiac repair and regeneration through the promotion of angiogenesis and supporting the survival of residual cardiomyocytes. In addition, our results from echocardiography, Masson's trichrome staining, and morphological analysis of mouse myocardium demonstrate that intramyocardial injection of Wnt-11-transduced MDSCs (Wnt-11 MDSCs), in combination with FGF2-coacervate, further enhances cardiac repair and regeneration when compared to non-transduced MDSCs delivered with FGF2-coacervate. Thus, Wnt-11 MDSCs delivered with FGF2-loaded coacervate can improve cardiac repair. These findings increase our understanding of basic biology of muscle-derived progenitor cell populations with enhanced cardiomyogenic potential, which may facilitate the development of new therapeutic approaches that merge the merits of stem cell therapy and biomimetic coacervate to improve cardiac repair and regeneration.

165. Marrow Infiltrating Lymphocytes (MILs): a Novel Adoptive Immunotherapy Approach for Metastatic Prostate Cancer Shows Measurable Tumor Specificity

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Introduction: Prostate cancer (PCa) is the most commonly diagnosed cancer in men and the 3rd-leading cause of cancer-related deaths in the United States. With metastatic disease (mPCa), 5-yr survival is 29%. Therefore, novel therapies remain a clinical priority in this setting. mPCa may be susceptible to autologous cellular immunotherapy. Sipuleucel-T, an autologous cell therapy for patients with mPCa, increased overall survival rates in phase III studies. Chimeric antigen receptor (CAR) T cell therapies directed at PSMA and PSCA are currently being developed in prostate cancer. Although promising, the overall efficacy and feasibility of these therapies remains unknown. Marrow infiltrating lymphocytes (MILs) are the product of activating and expanding bone marrow T cells. The bone marrow is a specialized niche in the immune system which is enriched for antigen

experienced, central memory T cells. MILs have been shown to confer immunologically measurable clinical benefits in patients with multiple myeloma. The bone marrow microenvironment has also been shown to harbor tumor-antigen specific T cells in patients with solid tumors such as breast, pancreatic and ovarian cancers. Herein, we sought to determine if tumor-specific MILs existed and could be expanded from the bone marrow in mPCa patients. **Methods:** Bone marrow samples were collected from hormone-naïve and castration-resistant mPCa patients (n=10) with varying amounts of bone marrow involvement. For a subset of patients (n=4), matched peripheral blood was also collected at the time of bone marrow aspiration. Utilizing a proprietary process, we activated and expanded MILs and peripheral blood lymphocytes (PBLs) from patient bone marrow and blood samples, respectively. T cell phenotypic markers (CD3, CD4 and CD8) were characterized by flow cytometry pre- and post-expansion. Tumor-specific T cells were quantitated in expanded MILs and PBLs using a previously described functional assay. Briefly, autologous antigen-presenting cells (APCs) were pulsed with lysates from prostate cancer cell lines and co-cultured with CFSE-labelled MILs or PBLs. APCs pulsed with myeloma cell line lysates or media alone were used as negative controls. Tumor-specific T cells were defined as the IFNγ-producing CFSE-low, $CD3^+$ population. **Results:** MILs were successfully expanded from every mPCa patient (n=10) with an average fold expansion of 346.4 (range: 29.1- 1625). Pre-expansion, the bone marrow T cell composition was 19.6% (7.8-29.5) $CD3^+$, 14.5% (7.5-26.2) $CD4^+$, and 6.6% (2.5-11.8) $CD8^+$. After activation and expansion, MILs were on average 91.2% (88.6-95.1) $CD3^+$ with an ~2:1 ratio of $CD4^+$:$CD8^+$ T cells [63.9% (37.2-80.2) vs. 27.2% (12.9-56.3), respectively]. Tumor-specific T cells were detected in all of the expanded MILs products (n=9). On average, 11.1% (1.25-40) of the total T cell repertoire were tumor specific for mPCa antigens in the MILs products. In contrast, matched PBLs expanded and activated from four patients demonstrated no measurable tumor-specific T cells. **Conclusions:** MILs were present and were expanded from all mPCa bone marrow samples tested. MILs from all patients contained functionally active tumor-specific T cells. In contrast, the corresponding PBLs failed to show any detectable tumor-specific immune recognition. As such, adoptive T cell therapy with MILs may be a viable novel therapeutic approach for patients with mPCa.

166. Evaluation of Non-Integrating RNA Measles Virus Vectors for Reprogramming of Human Hematopoietic Subsets

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Gene delivery has promised to provide an important method for the basic molecular medical science and the modern medicine including clinical gene therapy and regenerative medicine. In a viral gene delivery system of reprogramming method, considering the risk of the integrated genome insertion and low efficiency, an integration-free viral gene delivery system with safety and higher efficiency has become acceptable for generating induced pluripotent stem cell (iPSC). Recently, Sendai virus (SV) vector, a RNA-based virus vector, encodes the reprogramming factors with a mutant fusion (F) glycoprotein, can efficiently reprogram somatic cells without viral genome integration. Similar to SV, measles virus (MV) vaccine strain is safe and is a non-integrating single strand RNA virus replicated in the cytoplasm without genome intermediates. In addition, our newly developed-MV vector can be transduced into different cell types including resting lymphocytes by binding with several receptors including CD46, CD150 and Nectin-4. We developed a non-integrating gene transfer MV vector in which the F gene was deleted in order to avoid cell membrane fusion and prevent cell death. These MV vectors were further engineered to express transcription factors of OCT, KLF4, SOX2, L-MYC (OKSM) for reprogramming and EGFP reporter protein and named as MV-G-OKSM. The MV vector expressed EGFP without OKSM genes was also constructed and named as MV-G for checking the transduction efficiency to various cells. Hematopoietic cells were transduced with MV-G-OKSM for generation of iPSCs. The generated iPS-like colonies were picked up for further analysis. We first determined the MV binding surface receptors on the hematopoietic cell lineages. CD46, but not CD150 or Nectin-4, was expressed on the surface of most nucleated hematopoietic lineage cells. Next, we assessed whether MV could selectively transduce genes into the certain types of hematopoietic cells. Among the specific hematopoietic subsets, the transduction efficiency of MV-GFP was compared with that of SV-Azami-Green (SV-G) by using the same MOI. Furthermore, with the transduction of MV-G-OKSM vectors, we successfully generated high-quality iPSCs (referred to MV-iPSCs) with the similar morphology, pluripotency markers, karyotype and differentiation capacity as human ESCs. Notably, the MV delivery method of reprogramming required 45 minutes for gene transduction and formed iPS-like colonies between 10 to 14 days post MV-G-OKSM transduction. Moreover, we evaluated the reprogramming efficiencies of different hematopoietic subsets from human cord blood cells. The results showed the different reprogramming efficiencies among purified $CD34^+$ hematopoietic stem progenitor cells (HSPC), $CD3^+$ lymphoid cells and $CD33^+$ myeloid cells. iPSCs derived from the selective hematopoietic lineages expressed the pluripotency markers and had well differentiation ability. Considering the safe history of MV vaccines and carrying capabilities of multiple genes, our genetically engineered MV vectors would provide a new resource of RNA-based non-integrating gene transfer methods for reprogramming and would become a potential candidate for gene delivery method in the clinical immune therapy and regenerative medicine.

167. Epithelial Disruption Enables Human Airway Stem Cell Transplantation in Mouse Nasal Airways

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Introduction/Aim: Transplantation therapy using CFTR-competent cells has the potential to correct cystic fibrosis airway disease. One approach is to use human airway basal epithelial cells (HBEC) isolated from the CF patient's airways and correct *in vitro* using CFTR gene addition (or in the future, gene-editing). The cells are then delivered back to the airway to transplant, differentiate and normalise CFTR function to produce a therapeutic benefit. However, terminally-differentiated surface cells of the airway epithelium is a physical barrier to cell delivery. Providing access to the basement membrane and stem/progenitor cell region might increase therapeutic cell deposition and transplantation. The aim of this experiment was to test whether the removal of surface cells with polidocanol (PDOC) would enhance the level of HBEC transplantation in mouse nasal airways. **Methods:** Normal HBEC (LONZA, USA: CC-2540S) were seeded onto collagen-coated flasks, expanded to 75% confluency and transduced with a luciferase lentiviral vector at a MOI of 10. In normal female C57Bl/6 mice the nasal airway epithelium was exposed to a 4 µl aliquot of either PBS (control, n=10) or 2% PDOC (n=10) (Sigma-Aldrich). Two hours later, three 10 µl aliquots of HBEC-Luciferase were delivered into the treated nostril over 30 mins. Luciferase bioluminescence imaging (BLI; Xenogen, IVIS) was performed 1, 3, 5 and 8 weeks later to assess the persistence of luciferase gene expression produce by the HBEC. **Results:** No luciferase expression was present in the PBS pre-treated (control) mice. Mice that received PDOC treatment prior to delivery of HBEC-Luciferase showed significantly higher levels of nasal luminescence for up to 3 weeks compared to control mice ($p<0.01$, 2-way-ANOVA, Sidak's multiple comparison). **Conclusion:** These findings show that airway conditioning that disrupts epithelium integrity can improve *in vivo* luciferase expression from airway-delivered HBEC, suggesting that short-term functional cell transplantation can be achieved in living mouse airways. **Grant Support:** Studies supported by the USA CF Foundation (PARSON15GO) and the Cure 4 Cystic Fibrosis Foundation

168. Overexpression of FVIII Production in Placental Cells (PLCs) Increases Endogenous vWF

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Hemophilia A (HA) is an X-linked, recessive disorder caused by a deficiency in functional coagulation factor VIII (FVIII), which affects 1 in 10,000 males. In plasma, FVIII is bound to its carrier protein von Willebrand factor (vWF), a glycoprotein that plays an essential role in hemostasis by promoting platelet attachment to the injured vessel wall. Binding to vWF greatly enhances the stability and circulating half-life of FVIII, and also inhibits the uptake of FVIII by antigen-presenting cells (APC). We have recently demonstrated that human placental cells (PLCs) constitutively secrete low levels of vWF and functional FVIII, demonstrating that these cells possess all of the requisite machinery to efficiently produce and process these complex proteins. Here, we investigated the effects that FVIII upregulation had on PLC's constitutive vWF production. To this end, PLCs were genetically-engineering with a lentiviral vector (LV) encoding a bioengineered, codon-optimized FVIII transgene, designated mcoET3, under the control of the EF1α promoter. Following transduction with the mcoET3-encoding lentivector, PLCs expressed and secreted very high levels of functional FVIII, as measured by aPTT, and markedly upregulated endogenous expression of vWF, as determined by ELISA and immunofluorescence intensity using a GE IN Cell Analyzer 2000. Given the demands that expressing FVIII can place on the endoplasmic reticulum (ER) and secretory pathway, the upregulation of vWF following transduction with the mcoET3-encoding LV likely played a key role in the ability of PLCs to efficiently express and secrete high levels of FVIII without any signs of cellular stress. We next investigated whether vWF and FVIII colocalized within Weibel-Palade bodies (WPBs) within the transduced PLCs, as has previously been described for transduced endothelial cells. Somewhat surprisingly, analysis by transmission electron microscopy revealed that PLCs were devoid of WBPs, yet exhibited greatly expanded ER and highly active vesicle production. Despite lacking WBPs, immunofluorescence analysis revealed colocalization of FVIII and vWF within the transduced PLCs. In an effort to further increase FVIII secretion, we transduced PLCs with both the mcoET3-encoding LV and another LV encoding a truncated human vWF transgene. Evaluation of these dual-transduced cells demonstrated a decrease in FVIII production compared to PLCs transduced with the mcoET3-encoding LV alone, suggesting that endogenous vWF synthesis was already induced to optimal levels in PLCs following transduction with the FVIII LV.

169. Releasable Hydrogel Microspheres for Simplified T Cell Activation and Expansion Workflows

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Introduction Cost of goods concerns are driving increased focus on manufacturing unit operations for adoptive T cell therapies. T cell activation frequently employs antibody-coated magnetic particles which must be removed from the cell product, adding additional workflow steps and costs. We used a phase-change hydrogel biomaterial to develop a technology platform which potently activates and expands T cells and eliminates magnetic particles from the T cell activation process. ***Methods*** Ionotropic hydrogel microspheres of 9µm median diameter were prepared. In the presence of a chelating agent these microspheres rapidly depolymerize and enter liquid phase. Microspheres were functionalized with anti-CD3/CD28 antibodies to provide co-stimulatory activation signals. These functionalized microspheres are referred to as HyPer-ACT (Hydrogel Phase change-Enabled Release for Activation). CD3/CD28 Dynabeads and TransAct were used for comparison, according to manufacturer's instructions. Human CD3+ T cells or Human PBMCs were seeded (Day 0) at $0.5x10^6$ cells/well in 24 well plates (n = 3) in complete X-Vivo 15 medium with IL-2. 25 µL of HyPer-Act was added per $0.5x10^6$ cells in a single stimulation. Media addition was performed every 2-3 days. Following expansion, EDTA/PBS was added, and microspheres released from cells. Flow cytometry was used to assess activation and phenotypic markers. ***Results*** Addition of HyPer-Act induced expression of activation markers CD25 and CD69 in CD3+ cells (95% and 87% respectively on Day 2), similar to activation induced by Dynabeads and TransAct. After 9 days, HyPer-Act-activated cells underwent >7 population doublings, similar to growth observed for Dynabeads- and TransAct-stimulated cells. On day 9, cells were harvested. For HyPer-Act-expanded cells, cells were washed once during harvesting with a 2mM EDTA buffer to dissolve microspheres. Post-release, 95% of cells were recovered; no residual microspheres were observed. Following magnetic de-beading of Dynabeads-expanded cells, 85% of cells were recovered; residual magnetic beads were observed in the recovered cells after magnetic separation. CD3+ cells activated and expanded by HyPer-Act were evaluated for expression of CD45RA and CCR7. Cells on day 9 exhibited approximately 40% CD4+/CCR7+ expression and 55% CD8+/CCR7+, indicating early memory phenotypes. Of these cells, 9% of the cells were CD4+/CCR7+/CD45RA+ and 22% of the cells were CD8+/CCR7+/CD45RA+. A similar experiment was conducted with Human PBMCs. On Day 2 after HyPer-Act addition, 95% of the cells expressed CD25 and 85% CD69. On day 9, the cultures contained >99% viable CD3+ cells and the cells underwent >7 population doublings. Dynabeads-stimulated PBMCs were also activated after 48 hours and had a similar number of viable CD3+ cells and growth as HyPer-Act-expanded PBMCs. ***Conclusions*** This study demonstrates utility of HyPer-Act to potently induce T cell activation and expansion. No magnetic beads are present, thus simplifying activation, expansion and harvesting workflows within cell therapy manufacturing unit operations. Current development efforts are focused on cGMP manufacturing and use of this platform for improved yields of specific CAR-expressing T cell phenotypes.

170. GAIA-102: a Novel Natural Killer Cell Phenotype That Can Eliminate the Solid Tumors

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We recently developed a new, simple, and feeder-free method to generate highly active and expanded human NK cells from peripheral blood (Saito S, et al. Hum Gene Ther 2013;24:241-52), and have then investigated why our new type of highly activated and expanded NK cells, named as GAIA-102, are so effective to kill malignant cells. Importantly, we found that our GAIA-102 were involved in $CD56^{bright}$ population containing both $CD16^{high}$ and $CD16^{dim/-}$ subpopulations with high amount of perforin and granzyme B, unlike to any types of NK cells from peripheral blood that were $CD56^{dim}$ and $CD16^{high}$. Cell-sorting experiments demonstrated that $CD16^{high}$ population was highly specific to antigen-dependent cytotoxic cell (ADCC) activity that was peaked at 1~2 hours for tumor cell killing, whereas $CD16^{dim/-}$ population showed abundant, strong and long-lasting killing activity over 48 hours. As the results of the conjugate of these 2-different characteristics, GAIA-102 is highly effective to kill the any type of malignancies. More importantly, GAIA-102 is highly effective to kill some tumor cells in vitro and in vivo, including Raji, that are highly resistant to any types of NK cells activated by preexisting methods. GAIA-102 can also eliminate 3D spheroid of malignancies in vitro and human established tumors in mouse in vivo. We now just started GMP/GCTP production of this new and powerful NK cells and first-in-man clinical trials in use of GAIA-102 will be initiated on the early 2019.

171. Umbilical Cord Blood Mononuclear Cell Culture with Procell™ Microbiome Derived Peptide Stimulates Generation of Adherent Monocytic Cells Capable of Stimulating T Regulatory Cells

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Introduction: The peptide ProCell is derived from healthy microbiome and has previously been demonstrated to modulate several regenerative properties. While murine experiments demonstrated that ProCell is capable of generating monocytic cells capable of reducing disease in models of autoimmunity, it remains unclear whether similar immune modulatory cells may be generated from umbilical cord blood derived mononuclear cells (UCBMNC). **Methods**: UCBMNC where treated with ProCell and monocytic populations where extracted by CD14 Magnetic Activated Cell Separation (MACS). Immune modulatory activity of isolated cells was examined by coculture with allogeneic lymphocytes, as well as added to ongoing mixed lymphocyte reaction (MLR). Proliferation and cytokine production was assessed. Furthermore generation of T regulatory cells was examined by flow cytometry for FoxP3 and ability to suppress T cell proliferation stimulated by CD3/CD28. **Results:** Culture of UCBMNC in ProCell resulted in a population of tissue culture adherent expressing the

marker CD14 and possessing potent immune modulatory activity. Cells did not stimulate allogeneic T cell proliferation and actively inhibited ongoing MLR suggesting ability for active immunomodulation. MLR suppression was associated with augmentation of anti-inflammatory cytokines IL-10 and TGF-beta. Additionally, in vitro generation of active T regulatory cells was observed. **Conclusions:** Culture of UCBMNC with ProCell in a human in vitro system was effective at generating a monocytic population of immune modulatory cells. Immune modulatory monocytic cells potentially may be used in an allogeneic manner given lack of ability to stimulate allogeneic T cells. Generation of T regulatory cells suggested the possibility of infectious tolerance by ProCell treated PBMC, suggesting possibility of infectious tolerance induction.

172. DOR Activation Inhibits Hypoxia-Reperfusion Mediated Death of Human MSCs via Down-Regulating UPR and ROS along with Enhanced Anti-Inflammatory Effect

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Background: Hypoxia-reperfusion (H/R) emblems a plethora of pathological conditions which is potent in contributing to the adversities encountered by human mesenchymal stem cells (hMSCs) in post-transplant microenvironment, resulting in transplant failure. The role of D-Alanine 2, Leucine 5 Enkephaline (DADLE)-mediated delta opioid receptor (DOR) activation, well-known for its recuperative properties has been delineated herein, in terms of assuaging hMSC mortality under H/R insult. **Methods:** $CoCl_2$ mimicked the H/R conditions *in vitro* and DOR activation was mediated via DADLE. hMSCs loss of viability, Reactive oxygen species (ROS) production, inflammatory responses and disconcerted unfolded protein response (UPR) were assessed using AnnexinV/PI flow cytometry, fluorescence imaging, mitochondrial complex 1 assay, quantitative PCR, immunoblot analysis and ELISA. **Results:** H/R induced apoptosis of hMSCs was significantly mitigated by DADLE via modulation of the apoptotic regulators (Bcl-2/Bax) along with significant curtailment of ROS and mitochondrial complex 1 activity. DADLE concomitantly repressed the misfolded protein aggregation, alongside the major UPR sensors: PERK/BiP/IRE-1α /ATF-6, evoked due to the H/R induced endoplasmic reticulum stress. Undermined phosphorylation of the Akt signalling pathway was observed, which concerted its effect onto regulating both the pro and anti-inflammatory cytokines, actuated as a response to the H/R insult. The effects of DADLE were subdued by naltrindole (specific DOR antagonist) reaffirming the involvement of DOR in the process. **Conclusion:** Taken together these results establish the role of DADLE-induced DOR activation on improved hMSC survival. This signifies the plausible implications of the small molecule DADLE in cell-transplantation therapies and tissue engineering aspect.

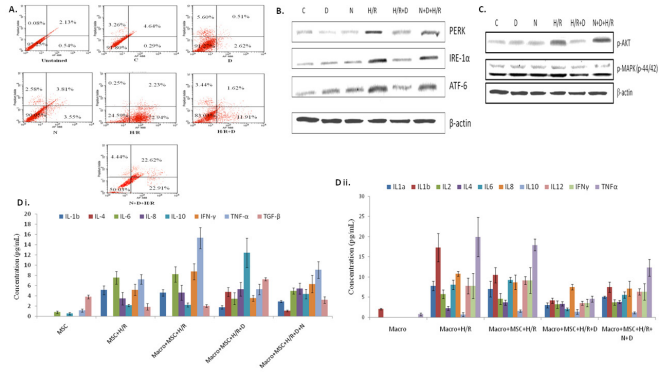

Fig. 1A: Cell viability of hMSCs under different treatments measured by Annexin V/PI using flow cytometry; **B:** Immunoblot of key UPR proteins under the different treatment conditions. C= untreated control, D= DADLE, N- Naltrindole, H/R- Hypoxia-reperfusion; **C:** Immunoblot of the activated levels of Akt and MAPK under similar conditions; **D i & ii:** Cytokine levels (ELISA) from murine and hMSC respectively under the following treatment conditions: MSC: untreated hMSC, MSC+ H/R: H/R treated hMSC, Macro+MSC+H/R: hMSCs co-cultured with murine-macrophages under H/R, Macro+MSC+H/R+D: hMSCs co-cultured with murine-macrophages under H/R with DADLE-mediated DOR activation, Macro+MSC+H/R+D+N: hMSCs co-cultured with murine-macrophages under H/R on naltrindole-treatment.

173. Humeral Factors Secreted by Human Bone Marrow Mesenchymal Stem Cells Enhances Steroidogenesis and Proliferation in Granulosa Cells

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Background: Premature Ovarian Failure (POF) is a devastating condition that affect up to 1% of women. POF is characterized by accelerated depletion of ovarian follicles and decreased oocyte quality, but the etiology remains unknown and treatment options are limited. Recently, we have launched an innovative clinical trial using human bone marrow mesenchymal stem cells (hMSCs) to treat POF (https://clinicaltrials.gov/ct2/show/NCT02696889). Autologous hMSCs are injected into one ovary followed by several clinical, imaging and serum hormonal evaluations. Our preliminary data demonstrate the ability of hMSCs to reinitiate ovarian steroidogenesis and to significantly increase serum estrogen levels up to one year post a single session of cell therapy. To further evaluate the impact of hMSCs on steroidogenesis, we used JC410 porcine granulosa cells that have been transfected with StAR (Steroidogenic Acute Regulatory) gene promoter-Luciferase marker cassette (JC410-StAR-Luc) as an in vitro model. StAR gene is crucial for transferring cholesterol to the P450scc enzyme and it expression reflects overall initiation and activation of ovarian steroidogenesis. **Hypothesis:** Our working hypothesis is that hMSCs secret useful humoral factors that activate granulosa cells and support ovarian folliculogenesis. **Methodology:** In JC410-StAR-Luc cell line, the expression of StAR gene can be sensitively monitored by

measuring luciferase expression. To mimic the in vivo co-presence of hMSCs and granulosa cell, we cultured JC410-StAR-Luc cell line in the presence of hMSCs conditioned media at two different concentrations: 1/2 and 1/3 for 48h at 37Oc AND 5% CO2. Ki67 proliferation marker and luciferase activity were measured using flow cytometry and spectrometry analysis respectively. Results were analyzed using student T-test. **Results:** The analysis of Luciferase activity showed a significant dose-dependent increase ($p<0.05$) in the induction of StAR expression by hMSCs conditioned media. We also found a significant a dose-dependent increase in the proliferation of JC-410-StAR-Luc exposed to hMSCs conditioned media as measured by the expression of Ki67 proliferation marker ($p<0.05$). **Conclusion:** Our study showed for the first time the ability of secretable factors from human bone marrow mesenchymal stem cells to enhance granulosa cell functions. Such novel cell therapy approach can be a new promising therapeutic tool to treat POF patients. Funding: University of Illinois at Chicago start-up research fund.

174. Cryopreservation Formulations and Container Systems Designed to Improve Storage and Transport of Cell and Gene Therapies

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Cell and gene therapy manufacturing and final products will often include cryopreservation of cells for storage and transport. The manufacturing process may require in-process frozen storage of viral vector product for example along with cryopreservation of the final product. Manufacturers of these therapies face many challenges when it comes to optimal cryopreservation formulation, processing, containment and transport as sub-optimal conditions can impact overall product quality. To help address the needs of the field, new cryostorage media and containers have been developed. Cryopreservation is common practice and often includes using formulations having DMSO prepared onsite or obtained pre-mixed. The EZ-CPZ and EZ-CPZ-ND [no dimethylsulfoxide, DMSO]) cryomedia are cGMP manufactured and made as concentrates for universal "ease-of-use". The media concentrates could be used directly for cryostorage or mixed with cells suspended in their preferred growth medium, usually a 1 to 1 ratio. Extensive studies done with hematopoietic, mesenchymal and epithelial progenitors showed that there were cell type differences, but there was high viability and recovery of greater than 80-95 percent when DMSO was in the formula at a 5 percent final concentration. Depending on the cells, the ND formula protected certain blood-derived mesenchymal and epithelial cells, with viability and recovery varying from 60 to 80 percent. The use of the ND formulation offers unique opportunities to limit or potentially remove DMSO and thereby reduce some of the known toxicities associated with DMSO. Associated with cryopreservation is the specific container used for storage and processing of the frozen material. Cryopreservation bags are commonly used for storage of cell-based materials for clinical applications and mainly for final product containment. These traditional containers have proven to be durable and compatible for cryopreservation and protection of cell therapies. These traditional containers are not optimal for in-process frozen storage for products like viral vectors which require further processing. Traditional cryocontainers lack the tubing required post cryostorage and thaw resulting in open processing leading to risk of contamination and product loss. Our newly developed FP-FLEX cryopreservation container and tubing have been designed to enable closed processing post storage eliminating the open handling step and resulting in reduced risk. The described products have been designed to address the unique cryopreservation storage and handling needs for cell and gene therapy manufacturing. The collective data and product design features support their utility to change and improve the logistics and shipping paradigm for the industry.

175. Serum Deprivation Accelerates Umbilical Cord-Derived Mesenchymal Stem Cells Differentiation towards Adipocytes and Osteoblasts

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Serum is an essential substance in cell culture media in which commonly produced from animals. It is known that serum contains nutrition and cytokines for cell growth, as well as adhesive molecules to assist cells attachment. Furthermore, serum also provides less stress environment for the cells and could act as protease inhibitor to protect their protein stability. Fetal bovine serum (FBS) has been widely used in cell culture due to its beneficial effects and ideal for the growth of various cell types. In this study, we found that FBS deprivation in umbilical cord-derived MSCs culture media was able to accelerate cell differentiation towards adipocytes and osteoblasts when induced by differentiation medium compared to cells cultured in media containing serum. MSCs were isolated from umbilical cord and cultured in Minimum Essential Medium (MEM) containing 10% Fetal Bovine Serum and 1% Antibiotic-Antimycotic up to 6th passage. The cells were cultured for 9 days in two conditions: serum-free medium (starved) and serum-contained medium (non-starved) and then continued to be further checked their MSC specific surface markers (CD 105, CD 73, CD 90) and ability to differentiate into adipocytes and osteoblasts. Differentiation of the cells was observed in two time points, 7th and 14th days for adipocytes and three time points, 7th, 14th , and 21st days for osteoblasts. The differentiated cells were then continued for histological staining and quantification assay. The immunophenotype data showed that in three different batches there was an unstable result of surface markers of UC-MSC. Batch 1: CD 90 91.48%, CD 105 97.05%, CD 73 99.09%, Batch 2: CD 90 89.87%, CD 105 67.25%, CD 73 83.56%, and batch 3: CD 90 94.65%, CD 105 87.75%, CD 73 92.76%. The <95% value of MSC surface markers could possibly be related to reduced stemness of the MSC. Starved UC-MSCs while induced by differentiation medium were differentiated into adipocytes on the 7th days, a week earlier than non-starved UC-MSC which is in the 14th days. A similar result in osteoblasts differentiation given by starved UC-MSC which differentiated in 14th days while it took 21 days for non-starved MSC. It could imply that the fate of MSC in serum-deprived condition is not only apoptosis but also promotes it to be differentiated towards adipocytes and osteoblasts when the microenvironments of differentiation are available in situ.

788. Non-Integrative and Small Molecule Strategies for the Generation of Expandable KDR+ Populations from CD34+ Human Umbilical Cord Blood

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Our goal is to utilize human umbilical cord blood (HUCB) cells, an established source of endothelial cells, to generate expandable stem cells for subsequent endothelial differentiation. Autologous human endothelial cells are of significant clinical interest for the treatment of vascular disorders. However, current strategies do not achieve stand-alone endothelial cell scalability for human applications. Other areas of regulatory concern in the field include genomic instability, low yields in generating target differentiated tissue, and the risk of tumor formation. To address these barriers and improve stability and safety, we generated putatively "dedifferentiated" stem cells from CD34+ HUCB tissue using small molecule and plasmid approaches. Generated stem cells were characterized in their dedifferentiated state for stem cell properties and clonality, and injected into mice for safety profiling. The generated stem cells were immunophenotypically classified based on surface markers ranging from most stemness to most differentiated. Dedifferentiated stem cell lines no longer expressed CD34, a key stem cell and progenitor marker, but to our surprise, expressed high and uniform levels of KDR, the earliest differentiation marker for definitive hematopoiesis and vasculogenesis. Generated stem cell lines consistently maintained 80-95% KDR+ expression levels, with extensive expansion potential (18-hour doubling time). KDR+ stem cell lines also expressed neuropilin-1 (NRP-1), which is expressed antecedent to CD34 and CD31 during development. When injected into NOD SCID gamma mice in a standard *in vivo* safety assay, KDR+ stem cells lines did not form tumors compared to controls over a duration of 80% of the mouse lifespan. Furthermore, when the small molecule is withdrawn as it would be upon injection in a therapeutic setting, KDR expression gradually decreased. During directed endothelial differentiation *in vitro*, stem cells lost KDR expression, and gained CD31 expression, the most sensitive and specific endothelial cell marker. Overall, we describe a novel non-integrative strategy for obtaining expandable dedifferentiated stem cells from CD34+ HUCB that express earlier markers notably KDR and NRP-1. The constitutive presence of the small molecule is a potential additional safety feature for clinical applications. Current work is focused on elucidating the roles of the small molecule and plasmid in the acquisition and maintenance of KDR+ stemness, and validating the fidelity of the final endothelial cell product.

Gene Targeting & Gene Correction I

176. *In Vivo* Gene Editing of the Murine Pcsk9 Locus Using Lipid Nanoparticle-Delivered Megatal mRNA

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MegaTALs are fusions between transcription activator-like (TAL) DNA binding domains and engineered meganucleases that can be reprogrammed to create double-strand breaks at a single genomic locus. Delivery of gene editing reagents to the liver via lipid nanoparticle (LNP)-encapsulated mRNA has emerged as a promising approach for *in vivo* genome editing, owing to the generation of robust yet transient protein expression in hepatocytes. To evaluate this approach, we reprogrammed a megaTAL to target the open reading frame of the murine *proprotein convertase subtilisin/kexin* type 9 (*Pcsk9*) gene. The *Pcsk9* gene serves as an ideal *in vivo* gene editing proof of concept target because of its enriched liver expression and clinically validated role in cholesterol homeostasis, allowing for facile evaluation of editing by tracking PCSK9 protein and cholesterol serum levels. The *Pcsk9* megaTAL demonstrates high *in vitro* editing activity, generating INDELs at greater than 80% of alleles when delivered to murine liver cell lines. To test the ability of the *Pcsk9* megaTAL to edit hepatocytes *in vivo,* we formulated megaTAL mRNA into LNPs and intravenously administered the formulation to mice via tail vein injection. The formulations were well-tolerated, causing minimal elevation of liver transaminases at 24 hours with no indication of abnormal liver pathology. Dose-dependent editing was observed across 0.3 to 3.0 mg/kg ranges of mRNAs, with INDEL rates of up to 60% at the PCSK9 locus in bulk liver, resulting in an approximately 80% reduction of serum PCSK9 protein. Moreover, a significant dose-dependent reduction in total cholesterol of up to 50% was observed, concomitant to the reduction in PCSK9 serum protein and efficiency of gene disruption. These proof of concept data demonstrate the high rates of editing and therapeutic potential the megaTAL platform can achieve when delivered *in vivo* by LNP-encapsulated mRNA to hepatocytes.

177. Comparison of Endonucleases and DNA Donor Template Delivery Methods to Improve Site Specific Gene Correction in Hematopoietic Stem Cells

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Site-specific correction of a point mutation causing a monogenic disease in autologous hematopoietic stem and progenitor cells (HSPC) can be used as a treatment of inherited disorders of the blood cells. Using endonucleases to induce a double-strand break (DSB) close to the point mutation, an exogenous homologous DNA donor template possessing the corrective base can be used by the targeted cells to repair the DSB by homology-directed repair (HDR). Sickle Cell Disease (SCD) is an ideal model to investigate the potential use of gene editing to correct a single point mutation at the beta globin locus. In previous work (Hoban et al. 2014), we showed efficient gene correction by HDR using Zinc Finger Nucleases (ZFN) of up to 20% in CD34+ cells from SCD donors *in vitro*; however, the rates of allelic disruption produced by non-homologous end-joining (NHEJ) were two to three times higher than HDR. Moreover, the corrective edits were significantly lower in the more primitive stem cells (10-30 fold), as shown by *in vivo* xenotransplantation experiments. The main goals of this study were to compare the two most commonly used endonucleases (ZFN and the Clustered Regularly Interspaced Short Palindromic Repeats [CRISPR]/Cas9) together with four different methods to deliver the homologous DNA donor template for HDR; and investigate if any endonuclease and DNA donor template combination could induce more efficient gene correction in long-term stem cells by achieving a greater HDR/ NHEJ ratio *in vitro* and *in vivo*. Four donor delivery methods were assessed along with ZFN in CD34+ cells: single stranded oligonucleotides, integrase defective lentiviral vectors, adeno-associated virus serotype 6 (AAV6) and adenovirus 5/35 serotype. High-throughput sequencing data showed that the ZFN plus AAV6 combination reached two to six times greater rates of gene correction (up to 40-50%) than any of the other donors. The ZFN plus oligonucleotide donor combination showed gene correction values consistent with what was previously observed (Hoban et al. 2014); however, the toxicity resulting from this combination remained the highest, even in absence of the ZFN. Most importantly, the ZFN plus AAV6 samples showed up to four times higher HDR/NHEJ ratios than the other donors. As the AAV6 and oligonucleotide donors provided the most promising results in the context of the ZFN, comparisons using CRISPR/Cas9 targeting the beta-globin gene along with these two donor templates were performed. Again, the AAV6 donor template led to greater rates of gene correction and higher HDR/NHEJ ratios than the oligonucleotide, and lower toxicity one day post-electroporation. *In vivo* experiments in NSG mice were carried out to compare engraftment capacity and gene correction frequencies in CD34+ cells treated with ZFN or CRISPR/Cas9, plus the AAV6 or oligonucleotide donor template. The rates of gene correction *in vivo* were two times greater using the AAV6 donor delivery than using the oligonucleotide donor. In all groups, gene correction frequencies measured *in vivo* were 4-9 fold lower than when measured *in vitro*, independently of the endonuclease or donor template used. These findings demonstrate that AAV6-mediated donor delivery was superior to use of a single-stranded oligonucleotide donor, but frequencies of gene correction in long-term stem cells remain lower than in short-term progenitor cells, independent of donor type.

178. Increasing HR-Mediated Genome Editing in HSPCs through Manipulation of DNA Repair Proteins

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Genome editing of hematopoietic stem and progenitor cells (HSPCs) is a promising technology for treatment of immune system disorders. Typically, targeted nucleases are used to introduce a DNA double-stranded break (DSB) at a specific sequence, with the outcome determined by the DSB repair pathway used. The two primary DSB repair pathways are non-homologous end joining (NHEJ) and homologous recombination (HR), resulting in gene disruption if NHEJ is used, or the copying of sequence information from an exogenous DNA template if HR is used. HR allows for a wider variety of outcomes, such as correcting a mutated DNA sequence, or the site-specific insertion of an additional DNA cassette, stimulating interest in skewing the repair pathway towards HR. However, NHEJ is the dominant repair pathway in human somatic cells, and HR is limited by factors only present at specific stages of the cell cycle. To overcome these limitations, we investigated whether manipulating the levels or activity of certain repair factors could promote HR-mediated repair in CD34+ HSPCs, including the most primitive subsets of this population. We tested a panel of drugs and mRNAs that code for repair proteins (native, mutant fragments and phospho-mimetics), including factors predicted to block NHEJ and stimulate HR. The factors were tested singly and in combination, in a hierarchy of experiments that included cell lines, primary human T cells, and HSPCs. The main editing protocol used in primary cells was based on ZFN mRNA electroporation and donor template delivery using AAV6 vectors, which we have previously shown to be highly effective at promoting HR-mediated editing in hematopoietic cells. These analyses revealed that inhibition of 53BP1, a master regulator of DNA repair pathway choice, was the most effective at stimulating HR. 53BP1 was inhibited with an engineered ubiquitin mimic, i53, which binds to 53BP1 and prevents its retention at DNA DSBs. In HSPCs, i53 reduced the 53BP1 foci formation and enhanced rates of HR-mediated gene editing by ~1.5 fold. Similar results were obtained at multiple loci and in primary T cells. In contrast, other attempts to inhibit NHEJ, including a small molecule inhibitor of DNA-PKcs and a Ligase-IV-destabilizing fragment (XR-Frag) did not increase HR rates, even when NHEJ was impacted. Combinations were also tested, revealing that i53 plus XR-Frag was synergistic, resulting in an overall HR

increase in HSPCs of 1.7-fold over controls. Finally, the impact of i53 on HR was also confirmed for the most primitive HSPC population (CD34+CD38-CD45RA-CD90+), and has no detrimental effects in methylcellulose colony-forming assays, indicating its usefulness for editing the most primitive and long-term repopulating cells. In summary, we have demonstrated that HR-mediated gene editing rates in HSPCs can be increased by the co-electroporation of a mRNA encoding i53, an inhibitor of 53BP1. Editing rates were enhanced in bulk CD34+ populations and all subsets, with no overt toxicity or impact on colony-forming potential. This suggests a simple technique for increasing HR repair outcomes in HSPC engineering.

179. Systemic Upregulation of Compensatory Disease Modifier Ameliorates Muscular Dystrophy Phenotypes *In Vivo*

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Most Mendelian disorders display clinical heterogeneity that cannot be solely explained by primary genetic mutations. This phenotypic variability is largely attributed to the presence of disease modifiers, which can exacerbate or lessen the severity and progression of the disease. Here, we sought to upregulate expression of a disease modifying gene using CRISPR/Cas9-based transcriptional activation system and assess the potential of this therapeutic strategy *in vivo*. As a proof of principle, we focused on congenital muscular dystrophy type 1A (MDC1A), which is caused by mutations in the *LAMA2* gene encoding Laminin α2, a protein that is important for maintaining stability of muscle fibers and myelination of neurons in peripheral nervous system. MDC1A patients suffer from the loss of muscle function and peripheral neuropathy. Transgenic overexpression of *Lama1,* encoding a structurally similar protein laminin alpha 1, ameliorates disease phenotypes in *Lama2*-deficient mice, suggesting its role as a protective disease modifier. Using a combination of three single guide RNAs we targeted the proximal promoter region of *Lama1* gene with a catalytically inactive *S. aureus* Cas9 fused VP64 transactivation domains. We systemically treated three weeks old dy^{2j}/dy^{2j} mice (animal model of MDC1A) with adeno associated virus serotype 9 encoding CRISPR/Cas9 components. Treated mice displayed robust upregulation of Lama1 in skeletal muscles and peripheral nerves in a dose-dependent manner. Muscle histopathology considerably improved, as evidenced by diminished numbers of infiltrating immune cells, reduced fibrosis, and dramatic decrease in myofibers with centrally located nuclei suggestive of normalized regeneration process in treated mice. Importantly, we observed significant improvement in the locomotion activity and specific tetanic force after upregulation of Lama1 expression. Collectively, our data demonstrate the feasibility and therapeutic benefit of CRISPR/Cas9-mediated modulation of a disease modifier *in vivo*, which opens up an entirely new treatment strategy for MDC1A patients. A similar approach can be applied to many disease modifiers, providing attractive therapeutic strategies for various inherited diseases.

180. Validation of an *In Vitro* CRISPR-Cas9 Off-Target Prediction Method in Rhesus Macaques

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The discovery of CRISPR-Cas9 has revolutionized targeted genome editing. Cas9 nucleases can be programed by guide RNAs (gRNA) to induce double stranded DNA breaks to modify genes of interest. However, Cas9 has been shown to tolerate up to 6 base pair mismatches between gRNA and unintended off-target sites. Accurate methods of CRISPR-Cas9 off-target site characterization are crucial prior to applying the system therapeutically. Computational algorithms, which rely on sequence similarity to the gRNA, have not been fully predictive in accurately predicting off-target sites. Whole genome sequencing to feasible depths cannot detect rare off-target events. Cell-based approaches to identifying off-target sites (OTs) require editing live cells and identifying off-target effects in their genomes. These methods are highly dependent on the efficiency of high-dose Cas9/gRNA delivery, making them less sensitive and unable to detect low frequency OTs in primary cells, for instance hematopoietic stem and progenitor cells (HSPCs), that are scarce and exhibit toxicity with Cas9 and gRNA overloading. These limitations have led to the development of CIRCLE-Seq (CSeq), an in vitro OTs detection method that can be performed directly on genomic DNA from relevant type of unedited cells. This bypasses the need for a Cas9/gRNA cellular delivery and allows for increasing concentrations of Cas9 and gRNA, thereby making the method extremely sensitive in detecting low frequency OTs. To date, CSeq has been validated only in cell lines, and not applied to in vivo samples from model animals followed long-term. We utilized our rhesus macaque model to ask whether top OTs identified via CSeq could be detected in blood cells collected from animals following autologous transplantation with CRISPR-Cas9-edited HSPC. We focused on gRNAs for AAVS1 and DNMT3A exons 3 or 19, sites utilized in our iPSC and/or transplantation models. Using pre-edited DNA from animal ZK48, we performed CSeq and identified 242 OTs for AAVS1, 59 for DNMT3A exon 3, and 66 for DNMT3A exon 19. The OTs with the highest rank based on CSeq read counts were the on-target site for both DNMT3A gRNAs, however, for AAVS1, the on-target site ranked only 4th. We compared our CSeq OTs for AAVS1 to OTs identified via a rhesus macaque modification of the most commonly used off-target algorithm (Hong et al, Mol Ther, 2017), using the reference standard rhesus macaque genome. The algorithm predicted 404 possible OTs. Only 61 of those overlapped with CSeq OTs. The top 10 algorithm-predicted OTs were examined via Sanger sequencing in iPSCs from another macaque and only 2 algorithm sites were found to be mutated. CSeq had only identified one and not the other of these two sites found mutated in iPSC. To confirm that this was not due to a polymorphism between the iPSC macaque and ZK48, we sequenced the pre-cut OT5 site in the iPSC and ZK48 and found no polymorphisms from the reference sequence. We performed CSeq on DNA from macaque ZL26 with gRNAs targeting AAVS1 and TET2. TET2 had 14 OTs identified by CSeq vs

195 by the algorithm. AAVS1 had 262 OTs by CSeq, 129 of which were previously found in CSeq on ZK48 DNA. ZL26 was transplanted with autologous HSPC edited with both AAVS1 and TET2 gRNAs, and had high levels of on-target edited hematopoietic cells post-engraftment. We intend to interrogate the validity of OTs identified by both algorithm and CSeq on blood samples from both ZK48 and ZL26 via multiplexed primer panels for the top sites for each gRNA. The resulting data will also provide insights into the variability in off-target effect between the macaques due to their unique sequence polymorphisms.

181. *In Vivo* RNA Targeting of Point Mutations via Suppressor tRNAs and Adenosine Deaminases

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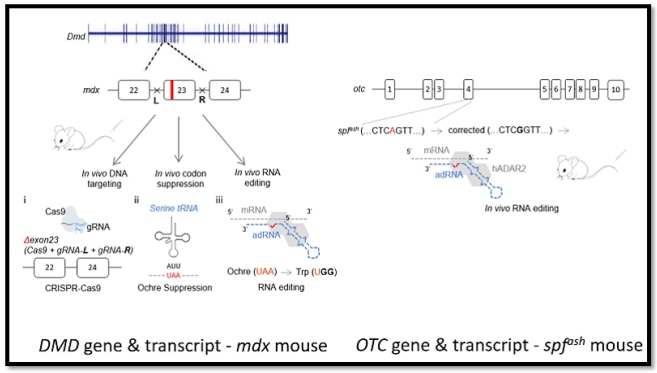
Point mutations underlie many genetic diseases. In this regard, while programmable DNA nucleases have been used to repair mutations, their use for gene therapy poses multiple challenges: one, efficiency of homologous recombination is typically low in cells; two, an active nuclease presents a risk of introducing permanent off-target mutations; and three, prevalent programmable nucleases typically comprise elements of non-human origin raising the potential of *in vivo* immunogenicity. Instead, approaches to directly target RNA with molecular machinery native to the host would be highly desirable. Towards this, we engineered and optimized two approaches, that of premature stop codon suppression via tRNAs as well as ADAR2 based RNA editing and for the first time demonstrated their activity in mouse models of human disease. First, we evaluated the suppressor tRNA and ADAR2 based editing in the *mdx* mouse model for Duchenne muscular dystrophy (DMD) which bears an ochre (TAA) stop site in exon 23 of the dystrophin gene. Specifically, by delivering modified endogenous tRNAs via adeno-associated viruses (AAVs), partial restoration of dystrophin expression was observed in the injected tibialis anterior (TA) muscle. Delivering the RNA editing enzyme ADAR2 and an associated guiding RNA (adRNA) via AAVs, we achieved modest TAA->TGG editing of dystrophin mRNA, leading to partial restoration of the protein. We also benchmarked our results with CRISPR based DNA editing. Next, we focused on the male sparse fur ash (*spf^{ash}*) mouse model of ornithine transcarbamylase (OTC) deficiency. The *spf^{ash}* mice have a G->A point mutation in the last nucleotide of the fourth exon of the OTC gene, which leads to OTC mRNA deficiency and production of a mutant protein. Adeno-associated viruses (AAVs) were used to deliver the human ADAR2 enzyme and adRNA to adult *spf^{ash}* mice resulting in correction of the mutation in 3-4% of the mRNA. We have demonstrated RNA targeting in two independent mouse models of human disease. Taken together, we believe that these approaches are robust, genomically scarless and potentially non-immunogenic as they utilize effector human proteins. We also note that an important consideration while considering targeting RNA versus DNA for gene therapy, is the necessity for long-term expression of effector constructs for the former due to the typically limited half-life of edited mRNAs. Moving forward, we anticipate this toolset, with progressive improvements, will have broad implications in both applied life sciences as well as fundamental research.



Pre-print: https://www.biorxiv.org/content/early/2017/10/28/210278

182. *In Utero* Base Editing of *Pcsk9* Leads to Sustained Edited Cells and Reduced PCSK9 and Cholesterol Levels

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Introduction. Individuals with nonsense variants in the proprotein convertase subtilisin/kexin type 9 (*Pcsk9)* gene are known to have reduced cholesterol levels and risk of coronary heart disease (CHD). Previous studies have demonstrated the feasibility of CRISPR-Cas9 mediated NHEJ to disrupt *Pcsk9* orthologs in hepatocytes *in vivo* postnatally. Base editing with the base editor, BE3, is a more novel form of CRISPR mediated genome editing in which a catalytically impaired SpCas9 unable to make double strand breaks (DSBs) is fused to the cytosine deaminase domain of the RNA editing enzyme APOBEC1. BE3 is able to make site specific C◊T or G◊A changes in the genome and in a safer fashion than CRISPR mediated NHEJ due to its inability to make DSBs. We have previously demonstrated postnatal i*n vivo Pcsk9* base editing up to one month. In the current study, we evaluate prenatal *in vivo* base editing of *Pcsk9* as a proof of concept model of *in utero* genome editing (IUGE) of a therapeutic gene. The rationale for IUGE is that many congenital genetic abnormalities result in significant morbidity shortly after birth and the potential benefits of targeting a therapeutic gene, including those involved in CHD, may be enhanced if the gene is corrected for the individual's lifetime. Furthermore, the small fetal size, accessible progenitor cells, and fetal immunologic immaturity provide the potential for edited cells to persist for a lifetime. **Methods.** BE3 and gRNA targeting a W159X mutation in the *Pcsk9* gene were delivered in an adenoviral vector (Ad.BE3.*Pcsk9*) to gestational day 16 Balb/c fetuses via vitelline vein injection. Adenovirus carrying GFP or an untargeted BE3 were injected as negative controls. DNA from liver, heart, brain, spleen, and lung was harvested at postnatal day (P) 1, 14, 30, and 90 and assessed by Surveyor and deep sequencing for *Pcsk9* and off-target editing. Serum was assessed for PCSK9, cholesterol and ALT levels. Statistics were performed with a Mann-Whitney U test. **Results.** In contrast to controls, fetuses injected with Ad.BE3.*Pcsk9*

demonstrated efficient stable hepatocyte editing (12% at P1, 16% at P14, 13% at P30, 12% at P90.) The most prevalent changes were W159X nonsense mutations with a small percentage of missense mutations. Off target analysis revealed less than 0.2% events and similar to those seen in control animals. The editing was hepatocyte specific with no editing in other organs. Prenatal *Pcsk9* base editing was associated with significant long-term reduction of serum cholesterol (27% reduction at P30 and 19% at P90) and PCSK9 levels (37% reduction at P30 and 50% at P90) without any significant deleterious effect on liver function. **Conclusion.** These results demonstrate the feasibility of *in utero* base editing of *Pcsk9*. They highlight the ability to achieve long-term edited cells with an associated desired phenotypic change. Although CHD will not be an initial target of IUGE, this study provides proof of principle for prenatal editing that could ameliorate genetic conditions that have significant neonatal morbidity/mortality.

183. Global and Tunable Suppression of Zinc Finger Nuclease and ZFP-Transcription Factor Off-Target Activity via Discrete Framework Substitutions

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Designed sequence specific nucleases and transcription factors offer the prospect of treatments for currently intractable conditions by enabling the modification or regulated expression of targeted loci in disease-relevant cells. A considerable challenge in the development of these agents, however, involves the need to minimize off-target effects while retaining therapeutically sufficient on-target activity. Among the platforms available to develop nucleases and transcription factors, strategies for addressing this challenge typically involve either labor-intensive cycles of redesign of the base-sensing interface, or a tradeoff between activity and specificity that may compromise on-target performance. To realize the full potential of gene-targeted medicines, approaches for optimizing specificity will be needed that both avoid these limitations, and are ideally simple, global, tunable and selective. To our knowledge, approaches that combine these features have not been previously described. In the work described here, we have addressed this issue in the context of designed zinc finger nucleases (ZFNs) and ZFP-transcription factors (ZFP-TFs) by developing a panel of single residue substitutions within otherwise invariant framework sequences that enable rapid optimization of specificity. These variants were developed by screening alternative residues at positions known or anticipated to nonspecifically contact DNA. Our studies proceeded in three stages. First, we examined substitutions within the zinc finger domain and identified a replacement - Arg(-8)Gln - that disrupts a highly-conserved phosphate contact and reduces nonspecific activity. Within the context of a well-characterized ZFN dimer, varying the number of fingers bearing this change provided an effective means for tuning total activity as well as on target preference. In the second stage of these studies, we examined substitutions within the Fok domain. In an analysis of 190 substitutions of 10 different DNA-proximal residues introduced into a previously characterized ZFN dimer[3], over twenty variants were identified that exhibited a broad spectrum of impacts on activity and specificity, including a single point mutant that reduced off-target cleavage 1000-fold while retaining full on-target activity. Finally, we combined approaches to generate nucleases targeted to the TCR alpha gene, and showed that the resultant ZFNs could introduce indels into the targeted locus in T cells at levels exceeding 99%, with little or no detectable off-target activity. In a parallel effort we have extended these studies to the optimization of ZFP-TFs. For our TAU program, we have shown that the introduction of three Arg(-8)Gln substitutions into a six-finger repressor enabled a 25-fold reduction in the level of off-target repression as gauged via microarray analysis. These results establish a new approach for optimizing ZFP specificity that should enable the development of highly specific ZFNs and ZFP-TFs for virtually any gene target.

[3] Nat Biotechnol. 2016 Apr;34(4):424-9. doi: 10.1038/nbt.3513.

184. Improving Gene Editing in Human Hematopoietic Stem Cells by Temporal Control of DNA Repair

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Gene editing of hematopoietic stem cells (HSCs) is a promising strategy for the treatment of monogenic diseases of the blood through site-specific correction of identified causal mutations. Our translational goal is to increase CRISPR/Cas9-mediated gene editing efficiency at the beta globin locus, in human HSCs, to ultimately correct the mutation responsible for Sickle Cell Disease. CRISPR/Cas9-mediated gene editing is achieved by inducing a double stranded break (DSB) near the mutation site, followed by employing innate cellular DNA repair mechanisms to fix the DSB. DNA repair pathways are temporally regulated. Error-prone non-homologous end-joining (NHEJ), which generally results in gene disruption, can take place in any phase of the cell cycle. However, precise homology-directed repair (HDR), which utilizes an exogenously-supplied donor template to correct the specific mutation, is restricted to S/G2 phases. Because the majority of HSCs are in G1 phase of cell cycle, NHEJ outcomes are more frequent than HDR. Thus, developing methods to improve the ratio of HDR to NHEJ is critical, especially for the correction of the sickle mutation, as disruption of the beta-globin gene could result in beta-null alleles. We hypothesize that temporal control of DNA repair can improve the ratio of HDR to NHEJ in human HSCs. We used a small molecule inhibitor of cyclin dependent kinase 1 (CDK1) to temporarily increase the proportion of cytokine-stimulated HSCs in S/G2 phases of cell cycle. Additionally, we used a modified version of SpCas9, termed hGemCas9, to decrease nuclease activity in G1 and M phases when HDR cannot occur. Our results demonstrate a significant increase in HDR/NHEJ ratio in primary

human HSPCs, compared to the control conditions, both *in vitro*, and after xenotransplantation of edited cells into NOD/SCID/IL2rg^{null} (NSG) mice. Importantly, transient cell synchronization did not affect hematopoietic potential as measured by human engraftment after competitive transplantation of synchronized and control cells into NSG mice. Furthermore, hGemCas9 had increased specificity, resulting in less off-target nuclease activity, compared to wtCas9. This strategy for improving gene editing outcomes in human HSCs has important implications for the field of gene therapy and may be applied to other diseases where increased HDR/NHEJ ratio is critical for therapeutic success.

185. Epigenetic Modifiers Targeted to the Genome of Hepatitis B Virus Induce DNA Methylation and Suppress Viral Replication In Vivo

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The estimated 257 million people chronically infected with hepatitis B virus (HBV) are at risk for complicating cirrhosis and hepatocellular carcinoma. High mortality resulting from chronic viral hepatitis, now exceeding that of infection with HIV-1, emphasizes the need to advance curative HBV therapy. Licensed anti-HBV treatments, including immune modulators and reverse transcriptase inhibitors, are modestly effective. Inability to inactivate the stable viral replication intermediate comprising covalently closed circular DNA (cccDNA) is the main limitation of current therapy. To address this shortcoming, we previously demonstrated that HBV-targeting transcription activator-like effector nucleases (TALENs) efficiently inhibit viral replication by mutating viral DNA sequences. However nucleases potentially carry a risk of mutating off-target sequences and cleaving host chromosomal DNA at sites of HBV DNA integration. Stable epigenetic modification of cccDNA is notionally a safer alternative that we have explored in this study. To generate epigenetic silencers, we fused four HBV-specific TALEs to combinations of protein modules that facilitate targeted DNA methylation. Constructs were designed to bind the plus (+) or minus (-) strand of the viral *surface (S), core (C)* and *polymerase (P)* open reading frames, and spanned the three CpG islands of HBV cccDNA. All four epigenetic modifiers reduced HBV surface antigen (HBsAg) secretion, a marker of viral replication, without causing measurable toxicity in transfected liver-derived Hih7 cells. S-targeting epigenetic silencers were evaluated in vivo using a murine hydrodynamic injection model of HBV replication. Antiviral action was compared to equivalent first generation KRAB-containing repressor TALEs (rTALEs). Using both epigenetic modifiers and rTALEs, serum HBsAg concentrations were reduced by up to 90%, intrahepatic viral RNA by 98% and circulating viral particle equivalents (VPEs) by 76%. To quantify methylation of viral DNA, mass array analysis was carried out. The TALE epigenetic modifiers caused an 8-fold increase in methylation of CpG island II, which was significantly higher than that observed when using the rTALEs. Viral DNA methylation effetced by the TALE epigenetic modifiers was also observed at sites that were up to 1 kb from the TALE cognates. Detailed evaluation of durability of methylation and inhibitory effects on viral replication is in progress using HBV transgenic mice. Sustained inactivation of HBV without causing mutation will be a valuable feature of anti-HBV epigenetic modifiers and the approach has potential for treatment of a disease of considerable global importance.

186. CRISPR-Skip: Programmable Gene Splicing with Single Base Editors

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Therapeutic exon skipping is a technique that typically utilizes antisense oligonucleotides (AONs) to exclude exons containing mutations from mature transcripts, thus creating a truncated protein that restores partial or full functionality and can ameliorate the symptoms of a disease. While this approach has been applied for correction of some monogenic diseases, the development of AONs is very costly and it does not provide a permanent correction, thus requiring repeated cycles of injections. Alternatively, gene editing has been used to force exon skipping; however, this process requires introduction of double-strand breaks (DSBs) into the genome, which are typically repaired by non-homologous end-joining and lead to creation of stochastic mutations. The unpredictable outcomes of this repair process, which include potential point mutations, deletions, truncations and chromosomal translocations, have raised safety concerns about its use in human subjects for therapeutic applications. One very promising approach to overcome these problems is the use of single base editors (SBEs), which introduce C>T or A>G mutations in DNA without creating a DSB. SBEs are still a nascent technology that have not been comprehensively characterized and, despite their wide-ranging potential, are utilized mainly for correction of SNPs. In this work we developed a novel exon skipping platform, which we named CRISPR-SKIP, that utilizes C>T base editors to induce skipping of the target exons by disrupting their splice acceptor sequence. Unlike AON approaches, CRISPR-SKIP introduces modifications in the genome and, therefore, correction is permanent and heritable. Since single base editors do not introduce DSBs, the repair outcome is not stochastic, thus decreasing the risk of introducing deleterious mutations. Our studies demonstrate that CRISPR-SKIP is broadly applicable in mammalian cells of different origins and we found that the rate of exon skipping is variable across targets, ranging from 2% to 32%. CRISPR-SKIP can be performed using a variety of Cas9 scaffolds and we developed a web-base software package to enable rapid and facile identification of potential target sites. Importantly, CRISPR-SKIP is multiplexable and can be used to skip multiple exons simultaneously to restore a reading frame. Finally, we compared CRISPR-SKIP with current state-of-the-art exon skipping using gene editing, and in each case, we achieved a greater degree of exon skipping with CRISPR-SKIP. Collectively, our results demonstrate a novel system for exon skipping that surpasses current state-of-the-art techniques and has wide applicability in biological engineering and biomedicine.

187. AAV Delivered CRISPR/Cas9-Mediated Platform to Knock-in and Express Promoterless Genes in the Lung to Correct Various Monogenetic Lung Deficiencies

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Over a hundred genetic diseases, including surfactant protein-B (SP-B) deficiency, lead to pulmonary distress and sometimes death. Many of these diseases are predominantly unresponsive to treatment other than organ transplantation. Therefore, we propose that a treatment approach could instead be used to correct the disease deficiency at the genetic level. We propose that a CRISPR/Cas9-based platform, delivered by adeno-associated virus (AAV), could insert genes in the lung leading to reversal of a deficiency and remedy of the disease. Here we describe a platform designed to knock-in genes in-frame with a genetic coding sequence that is specifically expressed in alveolar type 2 cells (AT2), replacing the stop codon for a 2a peptide followed by the gene of interest. This allows genes to be expressed in tandem with the host gene from its highly active promoter, exclusively in AT2 cells of the lung. It was decided that this expression system should be implemented to (a) eliminate the DNA size burden placed on AAV of packaging both the gene and a promoter, as well as (b) target expression to AT2 cells, which are dividing cells. This approach utilizes two separate single guide RNA (sgRNA) molecules, targeting Cas9 to introduce a double stranded break within close proximity to the gene stop codon and another directing Cas9 to cut further downstream, all of which facilitate insertion of the gene of interest via homologous recombination. The sgRNAs chosen cut the target DNA with 33.8% and 20.6% efficiency, respectively (figure 1). ifnar1 knock-out mice were injected intratracheally with an AAV vector encoding a human placental alkaline phosphatase (hPLAP) repair template, and separate AAV vectors expressing Cas9 and one of each of the sgRNAs. At 2 weeks post-vector administration, mice were euthanized and lungs were fixed and stained for hPLAP expression (figure 2). Despite delivery of a low dose of the repair template vector (4x109 vg), hPLAP foci were seen both grossly and histologically. These results suggest that this platform is capable of inserting and expressing genes from the genome of AT2 cells. Further studies will need to be performed to determine knock-in efficiency of this platform at higher vector doses, however, it can be suggested that this model could provide a method of inserting genes into host DNA for long-term expression from AT2 cells, which could be applied to the correction of various monogenetic lung deficiencies.

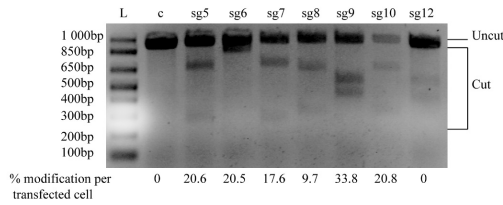

Figure 1. sgRNA cutting efficiencies determined by T7 endnuclease I assay following sgRNA transfection in MLE12 cells. T7 endonuclease I digests were analyzed by gel electrophoresis as well as quantitatively using a DNA 1000 chip with an Agilent 2100 bioanalyzer. C - no transfeciton control. sg5-10,12 - single guide RNA 5-10,12 in px601 plasmid backbone.

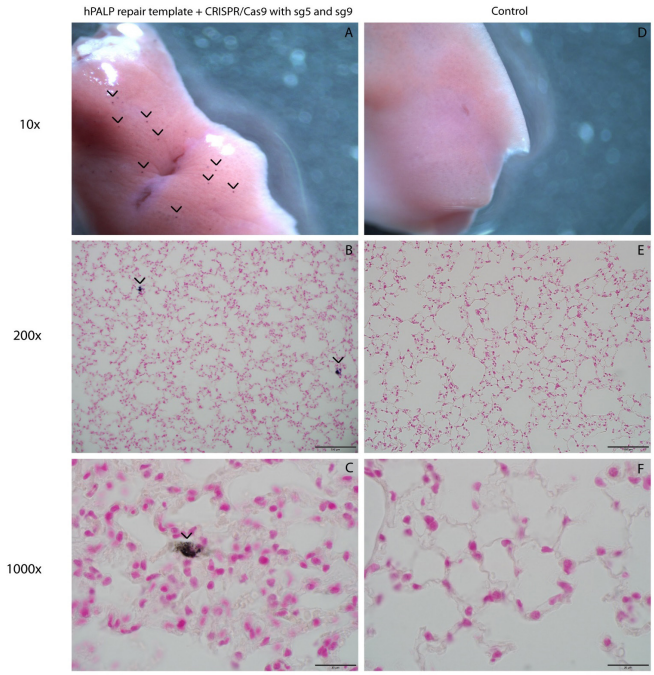

Figure 2. Alkaline phosphatase (AP) and H&E staining of *ifnar* -/- mouse lungs following intratracheal administration of AAV doses of 4x10^9 vg hPALP repair template, 1x10^{10} vg Cas9-sg5 and 3x10^{10} vg Cas9-sg9 (A-C) or no virus control (D-F). Mice were euthanized at 2 weeks post transduction. Lungs were stained for AP and visualized grossly (A, D), and tissues were then processed, H&E stained and visualized with microscopy (B,C,E,F).

188. Promoterless Targeting without Nucleases of Hyperactive Factor IX Corrects the Bleeding Diathesis in Hemophilia B Mice

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GeneRide™ is an AAV-based site-specific genome editing technology allowing life-long therapeutic benefits after a single injection at the neonatal phase in multiple disease models. GeneRide™ is nuclease-free, avoiding such hurdles as: nuclease delivery, immunogenicity, off target cleavage and on-target mutagenesis. In addition, GeneRide™ vectors are promoterless, thus reducing the risk of oncogene activation by rare

off-target integration. In GeneRide™, the promoterless coding sequence of a therapeutic gene is targeted by natural error-free homologous recombination (HR) into the *Albumin* locus. The expression of the therapeutic gene is linked to the robust hepatic Albumin expression via a 2A peptide. For the treatment of hemophilia B mice, we injected adult and neonatal mice with an AAV-DJ GeneRide™ vector coding for a hyperactive variant of human F9. In both groups, we demonstrated disease amelioration at doses as low as 1.5E12 VG/kg. The clotting time of treated mice was similar to wild type mice. In the relevant human ALB locus there are 2 major haplotypes covering 95% of the population. The haplotypes differ by 5 SNPs in the sequence corresponding to the 5' homology arm. We designed vectors with synthetic mouse haplotypes baring analogous mutations and found that GeneRide is largely unaffected by this haplotype mismatch. In conclusion, GeneRide™ obviates the need for either vector-borne promoters or the use of nucleases to induce integration and allows for safe and efficacious gene targeting for the amelioration of hemophilia B in both infants and adults.

189. Efficient CRISPR/Cas9-Mediated iIn Situ Correction of LAMB3 Gene in Keratinocytes Derived from Junctional Epidermolysis Bullosa Patient

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Junctional epidermolysis bullosa (JEB) is a recessively inherited skin disorder characterized by skin and mucosal blistering and by tissue separation along the lamina lucida of the cutaneous basement membrane zone (BMZ). The majority of JEB cases are caused by mutations in any of the three genes (*LAMA3*, *LAMB3*, or *LAMC2*) encoding the laminin 332 heterotrimer, the major adhesion ligand of basal epithelial cells expressed in the BMZ of the skin and mucous epithelia. Although the gene addition strategy based on viral delivery of an expression cassette for the therapeutic gene showed excellent results for JEB, the correction of genetic defect by homologous recombination (HR) into the native locus represents an attractive approach to obtain endogenous regulation of the introduced sequence. In this study, we propose to apply *S. pyogenes* CRISPR/Cas9 system to induce HR of a donor DNA template in the native locus for the correction of the vast majority of LAMB3 mutations. We designed CRISPR/Cas9 gRNA specific for intron II of *LAMB3* gene and a HR cassette carrying a promoterless splicible LAMB3 cDNA (therapeutic donor) engineered to be transcriptionally silent unless splicing event between the genomic exon 2 and the donor exon 3 occurs, upon HR event. JEB keratinocytes were co-transduced with Adenoviral vector carrying Cas9/gRNA and IDLV LAMB3 donor, and analyzed at a single cell level for *in situ* correction. Restoration of laminin 332 expression and related physiological adhesion, allowed us to *in vitro* positively select LAMB3-corrected cells. Molecular analysis on single isolated clones demonstrated an *in situ* monoallelic and biallelic integration of the therapeutic donor and a genuine splicing with the LAMB3 cDNA resulting in restored expression of laminin 332 heterotrimer. Laminin 332 expressing clones showed a morphology comparable to wt keratinocytes and an increased cell growth compared to JEB cells. Skin equivalents generated from two monoallelic-corrected clones were able to generate phenotipically normal human skin upon their grafting onto immunodeficient mice. Histological, immune-histochemical and molecular analyses on skin biopsies indeed demonstrated a functional reconstruction of the dermal-epidermal junction *in vivo*. In conclusion, our results provide clear evidences about the application of CRISPR-mediated homologous recombination to efficiently *in situ* rescue LAMB3 defects in keratinocytes derived from JEB patients, and pave the way for *ex vivo* preclinical application of this strategy to laminin 332 deficiency.

190. CRISPR Gene Correction for Severe Combined Immunodeficiency Caused by Mutations in *Recombination-Activating Gene 1* And *2* (*RAG1* and *RAG2*)

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The severe combined immunodeficiencies (SCIDs) are a set of life threatening genetic diseases in which patients are born with mutations in single genes and are unable to develop functional immune systems. While allogeneic bone marrow transplantation can be curative for these diseases, there remain significant limitations to this approach. Gene therapy using viral vectors containing a corrective transgene is being developed for some of these disorders; however, for other SCID disorders, such as those caused by genetic mutations in *RAG1* and *RAG2*, the transgene needs to be expressed in a precise, developmental and lineage specific manner to achieve functional gene correction and to avoid the risks of cellular transformation. In contrast to using viral vectors to deliver transgenes in an uncontrolled fashion, we are working towards developing CRISPR genome editing to correct the *RAGs* disease-causing mutations by precisely modifying the genome. CRISPR genome editing requires delivery of both the Cas9 nuclease and the targeting guide RNA (gRNA). The gRNA component can be generated in multiple ways, each with advantages and disadvantages. Here we compare the efficiency of editing, the on- and off-target repair profiles, and the innate immune stimulation of *RAG1* and *RAG2* gRNAs delivered as an *in-vitro* transcribed (IVT) single guide RNA (sgRNA), a chemically-synthesized sgRNA and a chemically-synthesized bipartite complex (crRNA + tracrRNA). Our results show that the chemically-modified sgRNAs and the Alt-R® bipartite crRNA + tracrRNA complex, delivered as a ribonucleoprotein (RNP) complex, enable the highest genome editing in human primary CD34+ hematopoietic stem and progenitor cells (HSPCs) with lowest toxicity. Additionally, we show that we can use the combination of CRISPR-Cas9 RNP, chemically modified gRNAs, and recombinant adeno-associated viral vector (rAAV) donor transduction to effectively target functional *RAG2* cDNA into the endogenous locus in human primary CD34+HSPCs. We will also present a summary of a comprehensive analysis of the

off-target events associated with the delivery of the synthetic *RAG1* and *RAG2* gRNA forms. The off-target profiles for each class of gRNA will be compared using the unbiased GUIDE-seq approach and quantified using rhAmpSeq™, a multiplexed, amplification-based, target enrichment next-generation sequencing (NGS) approach and finally the implication of our findings for therapeutic genome editing will be discussed.

191. Exploring the Messenger RNA Capping Code: CleanCap Co-Transcriptional Capping Allows Synthesis of Cap 0, Cap 1, Cap 2 and $^{M6}A_M$ Capped RNAs

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Messenger RNA (mRNA) therapy is an increasingly popular platform technology for expressing proteins in cells or *in vivo,* since there is minimal risk of insertional mutagenesis. mRNA transfection is being utilized to express proteins for genome editing (Cas 9, ZFNs and TALENs), protein replacement, vaccines and antibody expression. Exogenous mRNAs can be recognized as foreign by the innate immune system and one approach to avoiding innate immune responses is to mimic the structure of endogenous mRNAs. One important feature of mRNAs is the 5' cap structure. During RNA capping, Cap 0 (^{m7}GpppN) is formed as an intermediate. Methylation of the 2' position of the first cap proximal nucleotide forms Cap 1 (^{m7}GpppN$_m$N$\cdots$) which is found in all eukaryotic transcripts. In ~50% of transcripts, the 2' position of the second cap proximal nucleotide is also methylated to form Cap2 (^{m7}GpppN$_m$N$_m\cdots$). Another frequently found cap modification found in conjunction with Cap 1 (and potentially Cap 2) is N6-methylation of adenosine at the first cap proximal nucleotide (^{m6}A$_m$N$\cdots$). ^{m6}A$_m$ is the second most frequently found modification in mRNA. The role of mRNA cap structures remains to be fully elucidated. RNA viruses frequently encode methyltransferases that convert their Cap 0 structures to Cap 1. Deletion of this methyltransferase activity frequently leads to attenuation of these viruses. It is thought that IFIT proteins, and potentially other pattern recognition receptors, recognize Cap 0 structures as foreign, activating an antiviral state that represses translation. Thus, Cap 1, and potentially Cap 2 structures license endogenous mRNAs as "self" RNAs. The role of Cap 2 and ^{m6}A$_m$ is poorly understood since it has not been possible to produce such mRNAs synthetically at scale. A recent study suggests that ^{m6}A$_m$ caps may increase stability and translation while decreasing de-capping of mRNAs (Mauer et al., Nature 2016). Traditional co-transcriptional capping methods utilize ARCA (anti-reverse cap analog) and yield Cap0 structures which are immunogenic. ARCA capping results in low yields and poorly capped material (~70% capped). Post-transcriptional capping by Vaccinia virus capping enzymes can yield either Cap 0 or Cap 1 structures, but it is expensive and capping can be incomplete due to inaccessibility of structured 5' ends. Enzymatic capping also requires a purification step between transcription and capping. Previously, methods for producing Cap 2 RNAs have not been commercially available. Recently we developed a novel co-transcriptional capping method called CleanCap which can yield Cap 0, Cap 1, Cap 2, ^{m6}A$_m$ or un-natural caps. Capping with this method is reproducibly robust and highly efficient (90-99% capping), less expensive than enzymatic capping and is carried out in a "one pot" reactions without additional purification. Studies in THP-1 Dual monocyte cell lines indicate that these different cap forms alter the expression and immunogenicity of these mRNAs. Further studies are underway to characterize these RNAs *in vivo*.

192. Single Vector Transportation of CRISPR/Cas9 & Donor DNA for Homology Directed Repair of Canine Single Vector Transportation of CRISPR/Cas9 & Donor DNA for Homology Directed Repair of Canine Hemophilia B Reveals Higher Repair Efficiencies than Utilizing Two Vectors

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Hemophilia B is caused by mutations in the coagulation factor IX gene (FIX) and precise gene correction represents an alternative to treat this disease. Canine FIX and human FIX sequences display extensive sequence conservation, and possess 86% identity at amino acid level. The existing hemophilia B canine model contains a single point mutation in the catalytic domain of the canine coagulation factor IX (cFIX) gene leading to defective cFIX protein. To perform and analyze correction experiments we established HEK293 and the hepatocyte-derived Huh7 cell lines carrying the mutated cFIX locus stably integrated into the host cell genome. Based on the in vitro models, the Tet-on inducible cFIX-specific CRISPR/Cas9 system and the codon optimized donor were used to correct mutant cFIX through homology directed repair (HDR). For efficient delivery of designer nuclease and donor we produced a high-capacity adenovirus vector type 5 (HCAdV5) containing the Tet-on inducible cFIX-specific CRISPR/Cas9 system and a single-stranded adeno-associated virus type 2 (ssAAV2) vector containing a codon optimized cFIX donor. Moreover we designed a single HCAdV delivering both the Tet-on inducible cFIX-specific CRISPR/Cas9 system and the optimized cFIX donor for HDR. In addition, the allele refractory mutation system based quantitative PCR (ARMS-qPCR) analysis and the enzyme-linked immunosorbent assay (ELISA) were employed to measure HDR events on genotype and phenotype levels respectively. In the non-viral approaches performed in HEK293 cells we measured nuclease efficiencies of up to 43.25%. We found that the Tet-on inducible single vector applications in Huh7 cells resulted in up to 5,28% HDR efficiencies, higher compared to the two vector strategy. The ARMS-qPCR analysis showed that the efficiencies of co-delivery of the DOX inducible CRISPR/Cas9 HCAdV and the ssAAV2 donor vector in Huh7 cells revealed up to 3.95% HDR efficiencies and sufficient phenotypic correction of cFIX levels measured by ELISA. Since HDR efficiencies were increased for the single vector non-viral approach, we are currently exploring a single HCAdV carrying CRISPR/Cas9, gRNA unit and the codon optimized donor. We conclude that the combination of HCAdV5 and ssAAV2 for delivery of required HDR tools holds great promise for correction of mutant FIX in hemophilia B.

193. Modeling Epigenetic Diseases by CRISPR-Mediated Deposition of Methyl Cytosines and Lysines

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While gene therapy has traditionally focused on correcting DNA sequence-level alterations, there is a growing class of disorders whose disease manifestations strongly correlate with chromatin-level alterations. These "Epigenetic Diseases" display several features not detected at the sequence level including DNA methylation, aberrant histone post-translational modifications, and large-scale changes in chromatin accessibility. We posit that understanding the mechanisms of chromatin-associated disease states will significantly expand the utility gene therapy to target this increasingly appreciated class of epigenetic diseases. To model and decipher the rules that determine the stability of DNA methylation and its relation to chromatin-mediated gene expression we are focusing on *FRM1*, a gene that when epigenetically silenced leads to Fragile X syndrome. Epigenetic silencing of *FMR1* expression is associated with an expansion of a CGG trinucleotide repeat, increase DNA methylation and histone H3K9 tri-methylation of its promoter. To test the hypothesis that combinatorial assemblies of DNA methylation and H3K9 tri-methylation domains lead to a stable repressive state across mitotic cell division, we are using CRISPR/dCas9 to recruit DNA and histone methyltransferases to the murine *Fmr1* locus. We have observed a robust and sustained repression of Fmr1 when repressor complexes are co-recruited in the presence of histone deacetylase enzymes suggesting substrate availability is rate-limiting in epiallelic switching. The effect of DNA Methyltransferase 3A and 3L recruitment on the deposition of methyl cytosines will be presented, and its role in reinforcing a cellular pathological state will be discussed.

194. High Fidelity Genome Editing with a Novel Mutant HIF1 Cas9

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The CRISPR/Cas9 system demonstrates unparalleled editing efficiency in a broad range of host species and cell types but suffers from concerns related to target site specificity. Cleavage of genomic DNA at sites with an imperfect match to the guide RNA (gRNA) can occur as an undesired off-target effect (OTE). The magnitude of these OTEs is highest using expression-based systems like plasmids where the gRNA and Cas9 are highly expressed for several days or more. Use of chemically-modified synthetic guide RNAs delivered into cells pre-bound to recombinant Cas9 protein as a ribonucleoprotein (RNP) complex offers a "fast on, fast off" approach that maintains high on-target editing while reducing cleavage at off-target sites, however OTEs can still occur. To address this problem, Cas9 variants have been described where amino-acid contact sites with the substrate DNA were selectively mutated, reducing substrate affinity and at the same time reducing off-target activity. However, these mutants also significantly reduce on-target activity, particularly when used in RNP format. IDT has developed a novel mutant HiFi Cas9 protein that was evolved to reduce off-target gene editing while maintaining on-target potency. This mutant is particularly effective when used with RNP delivery, giving high efficiency genome editing with a reduced risk of OTEs.

195. Sensitive Changes in the Nuclear Import of pDNAs Can Be Detected with a Novel Macroscale Imaging Technique

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Many desired in vivo gene therapies are designed to target non-dividing cell populations, which means the nuclear membrane is a substantial barrier for non-viral delivery vectors. Previous research has demonstrated that specific DNA sequences can mediate the active nuclear import of plasmid DNA (pDNA) in non-dividing cells. These DNA nuclear Targeting Sequences (DTSs) can be universal, or cell-specific, but devising in vitro methods to screen for new DTSs can be a logistical challenge. There are many well established methods of quantifying in vitro transgene expression from fluorescence reporters, but most of them have time, cost, or sensitivity limitations. The most common method employs an inverted fluorescence microscope to take images of multi-well cell culture plates, but this relies on a small sample of representative images, or time consuming automated image stitching and subsequent image analysis. Plate readers can generate high-throughput fluorescence data of multi-well plates full of GFP positive cells, but data are derived from a limited amount of sampling areas within each well and do not always sample the entire cell population. We have developed a method using a fluorescence gel doc imaging station that allows us to rapidly quantify transgene expression in live cells. The advantages of our system is that it employs fairly common Western blot/gel doc imaging equipment, can be used with 6- to 96-well plates, non-invasively works with time course experiments, and sensitively images the entire population of cells within a well. We transfected Human Embryonic Kidney 293 (HEK293) cells using Lipofectamine 2000 or electroporation and a GFP plasmid that contains a universal DTS (pGFP-DTS). Four hours later, the cells were trypsinized and plated at densities of 5k - 300k per well in 6-well, 12-well, 24-well, or 96-well dishes, in triplicate. 24-hours after transfection, the plates were imaged with a Bio-Rad ChemiDoc MP imager using the green fluorescence LED emitter. Images were quantified using ImageJ. The gel doc imager was able to detect linear fluorescence changes in cell plating densities on the various multi-well plate formats. We also transfected two separate populations of HEK293 cells with either pGFP-DTS, or a plasmid variant that had the DTS sequence excised. Those transfected cells were plated onto wells of the same 24-well plate, at the same densities, and imaged with the gel doc imager at 4, 12, 18, 24, and 36 hours post-transfection. The imager was able to detect statistically different changes in GFP transgene expression between the two different transfections, as the pGFP-DTS plasmid led to higher transfection rates at the 4, 12, and 18 hour time points, before the population of HEK293 cells divided. These data were confirmed by fluorescence microscopy. Our data suggest that this methodology can be used to screen potential plasmid DTS sequences, even in dividing cells, in a high-throughput, accurate, sensitive, and cost-effective

fashion. Overall, identifying new DTS sequences that work in a variety of cell types could translationally improve in vivo gene therapies that target non-dividing cell populations.

Hematologic & Immunologic Diseases I

196. Saturated Mutagenesis Surrounding Beta-Globin Locus Identifies Novel Therapeutic Targets for Fetal Globin Induction and Treatment of Sickle Cell Anemia

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Sickle cell disease (SCD) is caused by a single mutation (E6V) at position 6 of the beta-globin chain. This mutation creates a hydrophobic patch that leads to polymerization of hemoglobin molecules and formation of sickle hemoglobin (HbS) fibers when deoxygenated. Affected red blood cells are rigid and prone to lysis, leading to anemia, acute chest syndrome, pain crises, and an array of other complications. Consequently, patients with SCD suffer significant morbidity and early mortality. Fetal hemoglobin (HbF), which inhibits the polymerization of HbS, is a key modulator of SCD severity. The onset of SCD symptoms occurs at around 5 months after birth, coinciding with the developmentally regulated fetal to adult globin switch. Patients with compound heterozygosity for SCD and hereditary persistence of fetal hemoglobin (HPFH) mutations within the beta-globin locus are typically asymptomatic. Therefore, therapeutic interventions that increase HbF are being investigated. Several groups are developing hematopoietic stem and progenitor cell (HSPC) therapies targeting BCL11A - a repressor of HbF, but clinical efficacy remains to be demonstrated. Here we report our efforts to identify a HSPC therapy that confines the genetic modifications to cis-elements in the vicinity of the beta-globin locus. This HSPC therapy is expected to produce persistent therapeutic levels of HbF with high specificity. We conducted a CRISPR-Cas9-mediated saturated mutagenesis screen that covered 320 kb region centered around the beta-globin locus. Human umbilical cord blood-derived erythroid progenitor HUDEP-2 cells with constitutive expression of SpCas9 protein were established and then transduced with a lentiviral library consisting of more than 27,000 guide RNAs (gRNAs). High HbF and low HbF producing cells were sorted and sequenced for integrated gRNA coding sequences. The enrichment of individual gRNAs was scored based on HbF expression. Over 300 gRNAs were found to be enriched in the high HbF pool. Hits were mostly concentrated at HBG, HBD, and HBB sites. Little enrichment was seen outside the 5'HS5 and 3'HS1 beta-globin locus boundary. gRNAs targeting known HPFH mutations at -110 and -200 regions of the HBG promoter showed strong enrichment. In contrast, gRNAs targeting transcription factor binding sites known to be important for HBG expression were depleted in the high HbF pool. In addition to known HPFH mutations, clustering of enriched guides in local subdomains revealed novel cis-elements that regulate fetal globin repression. The positive hits were validated in human CD34+ HSPCs electroporated with ribonucleoprotein (RNP) complex of SpCas9 and individual gRNA, and demonstrated elevated gamma-globin protein following *in vitro* erythroid differentiation. Editing in long-term hematopoietic stem cells (HSCs), as well as the impact on HSC function and erythropoiesis *in vivo* will be addressed in further engraftment studies in a xenotransplant model. In conclusion, novel HbF-inducing elements have been identified at the beta-globin locus using a CRISPR-Cas9-mediated saturated lenti-screen. gRNAs targeting these cis-regulatory elements can be developed to edit CD34+ HSPCs efficiently and generate erythroid progeny with high levels of HbF expression as a potential treatment of SCD.

197. Therapeutic Gene Editing for ß-Hemoglobinopathies by Means of Talen Mediated Fetal Hemoglobin Induction

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Hemoglobinopathies, including sickle cell disease (SCD) and β-thalassemia, are the most common single-gene disorders in the world. Importantly, hemoglobinopathy patients who also carry gene variants that induce expression of fetal (γ) hemoglobin often exhibit a less severe phenotype. Recent work has shown that the *in vitro* introduction of small deletions in the promoters of the $^{A}\gamma$ and $^{G}\gamma$ globin genes, including a previously described 13bp deletion in the promoter of the $^{A}\gamma$ globin gene (-102 to -114), can induce increased levels of fetal hemoglobin. Mounting evidence points to this region as a key site for binding of transcription complexes that mediate repression of fetal hemoglobin expression. We tested the capacity of TALENs targeting the 13 bp deletion region to disrupt the $^{A}\gamma$ and $^{G}\gamma$ promoter regions within the *HBB* locus. In human mobilized peripheral blood CD34+ cells, transfection of TALEN mRNA resulted in indel generation at both the $^{A}\gamma$ and $^{G}\gamma$ hemoglobin loci as confirmed by deep-sequencing, T7 analysis and digital droplet PCR (ddPCR). Indel rates at both loci ranged between 50-70% consisting primarily of small deletions within the 13bp deletion region. Edited CD34+ cells cultured in erythroid differentiation media for 10-14 days increased expression of fetal hemoglobin with a near doubling in the frequency of γ-hemoglobin (+) ‹F-cells› by flow cytometry and a threefold increase in γ-hemoglobin protein as detected by HPLC to as high as 40%. Patients treated with hydroxyurea achieve therapeutic benefit with as little as 10% fetal hemoglobin expression. These

findings support a model in which TALEN-mediated indels in the γ-hemoglobin promoters are capable of de-repressing fetal hemoglobin in hematopoietic progenitor cells. To assess the engraftment potential and fetal hemoglobin production of TALEN-edited CD34+ cells *in vivo*, modified cells were transplanted into recipient NSG-W41 mice, a strain demonstrated to support human erythropoiesis and high rates of engraftment with minimal conditioning. An analysis of indel rates at 24 weeks post-transplantation demonstrated maintenance of approximately half of the indels detected in the initial modified CD34+ cell product. At the time of bone marrow harvest, human F-cell detection by flow was significantly higher in recipients of TALEN edited CD34+ cells compared to mock controls and this difference was maintained following harvest and *in vitro* differentiation. Lineage sorting and genomic analysis demonstrated that indels were present in multiple hematopoietic cell lineages indicating that edited CD34+ cells are capable of multi-lineage engraftment. To assess if indels were generated in a long-term repopulating stem cell (LT-HSC) population, secondary transplants with bone marrow derived from primary recipient mice were performed. At 9 weeks post-transplant, engrafted human cells within the bone marrow of secondary recipient animals had indel rates that were equivalent to primary donors indicating no selective disadvantage to edited cells. These findings demonstrate that TALEN mediated indels in the γ hemoglobin promoters of the *HBB* locus in mobilized CD34+ hematopoietic stem cells promote high levels of fetal hemoglobin expression and strongly support the conclusion that this approach may be sufficient to achieve therapeutic benefit in patients with SCD or β-thalassemia.

198. Hematopoietic Stem Cell-Based Gene Therapy Using Lentiviral Vectors to Treat Hereditary Pulmonary Alveolar Proteinosis

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Hereditary Pulmonary Alveolar Proteinosis (herPAP) is a rare inherited lung disease characterized by mutations in the genes encoding the granulocyte/macrophage-colony stimulating factor receptor (GM-CSFR) α- (*CSF2RA*) or β-chain (*CSF2RB*) and impaired alveolar macrophage (AM) differentiation and functionality. Subsequently, surfactant proteins and phospholipids are progressively accumulating in the intra-alveolar spaces causing respiratory insufficiency and high susceptibility to respiratory infections. So far, therapeutic options are extremely limited, as allogeneic hematopoietic stem cell transplantation (alloHSCT) or HSCT-based gene therapy (HSC-GT) remain problematic due to the preexisting lung disease. Recently, pulmonary transplantation of macrophages has been introduced as a new therapeutic strategy for herPAP. This intervention may resolve ongoing infections and stabilize the patient to allow for future alloHSCT or HSC-GT. Therefore, we established a lentiviral-based HSC-GT approach in a murine *Csf2rb*$^{-/-}$ model. We utilized a 3rd generation SIN lentiviral vector expressing *Csf2rb* and a GFP reporter driven by an elongation factor 1 α long (EF1) promoter yielding robust transgene expression on lineage negative bone marrow (lin$^-$) cells and thereof derived macrophages (MΦ) *in vitro*. Furthermore, lentiviral transduction rescued GM-CSF-dependent functionality in *Csf2rb*$^{-/-}$ lin$^-$ cells as demonstrated by restored GM-CSF-dependent colony formation as well as MΦ differentiation potential with restored GM-CSF uptake and STAT5 phosphorylation in differentiated MΦ. Intravenous injection of corrected lin$^-$ cells into lethally irradiated *Csf2rb*$^{-/-}$ recipients resulted in rapid restoration of the AM pool, with first Siglec-F$^+$/CD11c$^-$ progenitors being observable two weeks after HSCT and mature Siglec-F$^+$/CD11c$^+$ AM four weeks after HSCT. Of note, marked clinical improvement was observed twelve weeks after HSCT as indicated by decreased turbidity, protein and cholesterol levels in bronchoalveolar lavage fluid (BALF). Furthermore, concentrations of GM-CSF, M-CSF and MCP1 in BALF were normalized to wildtype levels. Histologic lung sections revealed a decrease of Periodic Acid Schiff positive material in mice receiving corrected cells. Additionally, improved lung parameters were observed as indicated by decreased densities in CT scans as well as higher inspiratory capacities and static compliance. Although the engraftment of gene corrected cells was rather low (1% in bone marrow), a pronounced clinical improvement was observed even 9 months after HSCT and in secondary transplants. Taken together, our data highlights the feasibility and efficacy of a lentiviral HSC-GT approach to herPAP, thus offering a new therapeutic approach to tackle this live-threatening and so far incurable disease.

199. Hemostatic Efficacy and Provocative Safety Studies of Factor IX Padua Gene Therapy in Hemophilia B Dogs

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Introduction: Hemophilia B (HB) is an X-linked bleeding disorder due to a deficiency in factor IX (FIX). Previous HB gene therapy (GT) trials reported that the efficacy of liver directed AAV vectors was limited due to a dose dependent cellular immune response against the capsid. We recently demonstrated that the use of the hyperactive variant, FIX Padua, allowed for a 4 fold lower vector dose compared to earlier trials with wild type (WT) FIX, but achieved 5 fold higher FIX activity levels and limited the cellular immune response. FIX Padua is due to a single amino acid substitution (R338L), which results in an 8 fold increased specific activity. Though FIX Padua has been adopted by all current GT trials for HB, its hemostatic efficacy and immunogenic risk have not been rigorously defined. **Results:** We have undertaken long term studies in HB dog models expressing canine (c) FIX Padua after both liver and muscle directed AAV GT. We report a complete abrogation of the bleeding phenotype in HB dogs after 1 to 3 x 10^{12} vg/kg AAV-cFIX-Padua GT (n = 5 muscle directed; n = 4 liver directed). Prior to GT, these dogs cumulatively had 24 bleeds in 173 months;

post GT, there have been no bleeds, with a cumulative follow up of 493 months. We observe sustained FIX activity levels of 4 - 175% and specific activities of 8-12 fold cFIX WT; follow up ranges from 3 - 8 years. We also observe that recombinant cFIX Padua displays a similarly increased specific activity compared to WT as the human orthologues. In a 20 amino acid screen at position 338, we observe that most substitutions increase the specific activity of both hFIX WT and cFIX WT, while R338L is one of the most active substitutions. These biochemical similarities support the use of cFIX Padua as a highly relevant model as similar mechanisms are likely responsible for the increased specific activity of both orthologues. An additional dog received only 9 x 10^{11} vg/kg of the same muscle directed vector. This resulted in sustained FIX activity of 1%, though undetectable antigen levels. Prior to reaching 1% activity, he had 14 bleeds in 15 months; subsequently, he did not bleed (31 months observation). The ability of 1% activity provided by FIX Padua to prevent all bleeding supports its *in vivo* hemostatic efficacy. Even this low antigen level was sufficient to maintain immune tolerance despite treatments with cFIX WT. The concern that low antigen levels trigger inhibitor formation in mice is not supported by our data in outbred models. In inhibitor-prone HB dogs, where a single injection of cFIX protein results in the formation of inhibitors against cFIX, we observe that both liver directed (n = 3) and muscle-directed (n = 2) GT with cFIX Padua induced immune tolerance. This immune tolerance was maintained despite widely spaced challenges with cFIX WT. Muscle expression does not benefit from the same bias towards immune tolerance as liver expression. As such, these experiments represent the most challenging scenario, combining an inhibitor-prone model with a susceptible target tissue. We also observe in 2 inhibitor prone dogs with preexisting inhibitors, liver directed GT resulted in sustained inhibitor eradication. **Conclusions**: cFIX Padua recapitulates many of the biochemical features of the human orthologue. Expression of cFIX Padua prevents bleeding in HB dogs, with as little as 1% activity demonstrating efficacy. Both liver and muscle expressed cFIX Padua demonstrated similar immunogenicity as cFIX WT. These studies reinforce the ongoing use of FIX Padua in liver directed GT clinical trials and highlight the potential of muscle directed approaches.

200. Reactivation of Human Gamma-Globin in Adult Beta-YAC Mice Transplanted with HDAd5/35++-CRISPR/Cas9 Edited Hematopoietic Stem Cells

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Disorders involving β-globin gene mutations, mainly sickle cell disease and β-thalassemia, become symptomatic postnatally as fetal γ-globin expression from HBG genes (HBG1 and HBG2) declines and adult β-globin increases, thereby shifting red blood cell (RBC) hemoglobin from the fetal (HbF) to adult form. In hereditary persistence of fetal hemoglobin (HPFH), a benign genetic condition, mutations attenuate γ-to-β switching, causing high-level HbF expression throughout life thus alleviating the clinical manifestations of hemoglobinopathies. Recently, HBG1/2 promoter editing using CRISPR/Cas9 to recapitulate HPFH mutations has been performed in human CD34+ cells that were subsequently differentiated into erythroid cells either in vitro or after engraftment in NSG mice. While these studies demonstrated reactivation of HbF, this cell model does not adequately reproduce erythroid maturation and globin switching and displays a high level of HbF expression in adult cells without gene editing. We therefore used mice that carry 248kb of the human β-globin locus (β-YAC mice) and thus accurately reflect globin switching. We employed a new helper-dependent adenovirus vector platform (HDAd5/35++) expressing a HBG1/2 promoter-specific CRISPR/Cas9 to disrupt a recently identified binding site for an isoform of the HBG gene suppressor BCL11A. Transduced HSCs from β-YAC mice were transplanted into irradiated recipients and human HbF expression was measured in RBCs by qRT-PCR, flow cytometry, and HPLC. The engraftment levels of transduced HSCs were close to 100%. Ten to thirty percent of RBCs expressed human HbF. Simultaneously, levels of human β-globin declined indicating a reversal of globin switching. Missmatch-sensitive T7E1 nuclease and Illumina MiSeq analysis of genomic DNA from bone marrow cells showed efficient genetic knockout of the BCL11A binding site. Analysis of cellular composition in bone marrow, spleen, and blood did not show negative effects of HBG-promoter editing on erythropoiesis and erythroid maturation. The percentage of HbF-positive cells (~10-25%) was maintained in secondary recipients over 16 weeks indicating that the HDAd5/35++ vector targeted long-term repopulating mouse HSCs. Our study demonstrates, in an adequate animal model, that HDAd5/35++-CRISPR/Cas9 mediated HBG promoter editing is safe and therapeutically relevant.

201. CRISPR-Based Therapy for IPEX Syndrome as a Model of Genetic Autoimmunity

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CRISPR-based gene correction in hematopoietic stem and progenitor cells (HSPCs) has the potential to treat a wide variety of genetic and acquired diseases. Gene corrected HSPCs can be used for autologous transplantation, circumventing the need for HLA-matched donor transplants. Here we propose a CRISPR-based gene correction strategy to treat IPEX syndrome, the prototype of genetic autoimmunity. IPEX is a life-threatening x-linked disorder caused by mutations in the *FOXP3* gene. *FOXP3* mutations lead to dysfunction of T regulatory cells (Tregs) and autoimmune manifestations. Due to the widespread distribution of *FOXP3* mutations throughout the gene, we designed a gene repair strategy to insert a cDNA encoding wildtype FOXP3 protein into the mutated gene locus. This site-specific gene editing strategy allows for maintenance of regulated gene expression in patient cells independent of the location of the downstream mutation. To gene edit *FOXP3*, we used a CRISPR RNP combined with an AAV6 packaged donor DNA template, and showed that the system effectively targets *FOXP3* in HSPCs. We then demonstrated that gene edited HSPCs can be enriched to over 95% purity by cell sorting or magnetic bead enrichment. As a proof-of-principle, we also edited primary Tregs and T effector cells, the two major cell types that express *FOXP3*. This allowed us to demonstrate that *FOXP3* is expressed after editing in both healthy donor and IPEX patient cells. Furthermore, we showed

that gene edited Tregs are functional using *in vitro* Treg suppression assays. Lastly, we examined the *in vivo* function of gene edited HSPCs, and observed long term engraftment of the edited cells in NSG mice. This study supports a CRISPR-based autologous transplant strategy to treat IPEX syndrome, and further underscores the potential of gene editing treatments for other genetic immune diseases.

202. Therapeutic Induction of Fetal Hemoglobin by Highly Efficient Cas9-Mediated BCL11A Enhancer Disruption in Sickle Cell Disease Hematopoietic Stem Cells

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Sickle cell disease (SCD) is a severe monogenic blood disorder resulting in excess morbidity and mortality. Curative therapy, namely allogeneic hematopoietic stem cell transplant, carries substantial risk and is only available to a small fraction of the millions worldwide affected. Reactivation of fetal hemoglobin (HbF) could prevent deoxyhemoglobin polymerization, the fundamental molecular abnormality causing the clinical sequelae. Here we investigate a strategy applying CRISPR-Cas9 genome editing to permanently modify human hematopoietic stem cells (HSCs) to enable durable autologous production of therapeutic levels of HbF. We target the erythroid enhancer of *BCL11A*, where sequences that harbor common naturally occurring genetic variants associated with SCD clinical severity are necessary and sufficient for erythroid *BCL11A* expression but dispensable for non-erythroid expression. First we screened a set of 20 guide RNAs targeting the functional core of the +58 *BCL11A* enhancer for efficient sequence disruption by nonhomologous end-joining. We introduced Cas9:sgRNA as a ribonucleoprotein (RNP) complex by electroporation to G-CSF mobilized peripheral blood CD34+ hematopoietic stem and progenitor cells (HSPCs) from healthy donors. We overcame variability in editing efficiency by use of chemically synthesized and modified guide RNAs, Cas9 protein with additional nuclear localization sequences, and optimized electroporation buffer. We identified a lead guide RNA able to induce highly efficient editing with ~90% on-target indels, which produced robust disruption of erythroid BCL11A expression and derepression of γ-globin. Clonal analysis showed that even 1 bp indels around the cleavage site were sufficient for HbF reactivation. Specificity was evaluated by CIRCLE-seq, a method to define genome-wide target sequences susceptible to RNP cleavage in vitro. Amplicon deep sequencing of the top 16 possible off-target sites from edited CD34+ cells did not reveal any off-target editing with limit of detection 0.1% allele frequency. We found that edited CD34+ HSPCs contributed to marrow engraftment and multilineage hematopoiesis in immunodeficient NBSGW mice after 16 weeks at similar levels as unedited cells. Edited engrafting cells were competent for secondary transplantation. Long-term engrafting cells maintained >90% on-target indel frequency. Gene edited bone marrow derived erythroid cells expressed ~60% γ-globin (of total human β-like globin) as compared to <5% in unedited cells, as well as ~40% HbF as compared to <5% in unedited cells. We observed a similar degree of highly efficient *BCL11A* enhancer editing, long-term multilineage engraftment, and in vivo HbF induction in plerixafor-mobilized peripheral blood CD34+ HSPCs from SCD patients. Reticulocytes derived from long-term engrafting edited SCD CD34+ cells were resistant to in vitro sickling. In summary, we have identified highly efficient conditions to produce HbF-inducing *BCL11A* enhancer genome edits ex vivo in CD34+ HSPCs from SCD patients in a manner that neither induces apparent genotoxicity nor impairs long-term repopulating HSC function. *BCL11A* enhancer editing approaching complete allelic disruption appears to be a feasible strategy for durable HbF induction for the β-hemoglobinopathies.

203. Efficient Ex Vivo γ-Globin Gene Transfer in Human Thalassemic CD34+ Cells Using an Integrating Hybrid Adenoviral Vector System

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Current ex vivo approaches for gene therapy of hemoglobinopathies rely on the use of self-inactivating lentiviral vectors (SIN-LVs). Although successful at some degree and safe so far, these approaches are challenged by i) the relatively low transduction efficiency, due in part to the limited SIN-LV transgene accommodating capacity which translates into suboptimal titers ii) the semi-random integration of LVs with preference for active genes, generating a non-negligible risk for transformation, especially in the context of vectors carrying very powerful enhancers as the globin vectors. We propose an alternative platform for gene therapy of hemoglobinopathies to effectively address some of the current challenges. An integrating, CD46-targeted, high-capacity (30kb) adenovirus HDAd5/35++ vector carrying a full-length human γ-globin gene, was used in combination with a second HDAd5/35++ vector incorporating an enhanced Sleeping Beauty transposase (SB100x) to mediate random transgene integration. Vectors were produced at a titer of ~$1x10^{13}$vp/ml. This hybrid vector system was tested in human CD34+ cells from thalassemia-major patients previously enrolled in mobilization trials. After overnight transduction, at a total MOI of 500 vp/cell, $1x10^6$ $CD34^+$ cells were transplanted into NSG mice conditioned with Busulfan 100mg/kg (equivalent to 8mg/kg in humans). Multilineage reconstitution was achieved in all mice and importantly, there was no significant difference on the levels of engraftment between untransduced (25%) and transduced CD34+cells (18%), suggesting that HDAd5/35++ transduction does not negatively affect the engraftment potential of $CD34^+$ cells. Because the NSG mouse model does not support human

erythropoiesis and in order to determine the expression levels of γ--globin in human erythrocytes, mice were sacrificed 10 weeks after transplantation and hCD45$^+$ cells were isolated from the bone marrow and seeded in methylocellulose and in erythroid differentiation culture (EC) or/and infused in secondary recipients. The HbF expression in BFU-Es from the transduced samples was higher than in BFU-Es from the untransduced samples, as reflected both by the % of HbF expressing enucleated red cells (62% vs 11%, respectively) and the mean fluorescence intensity of HbF expression (345 vs 154, respectively). Importantly, the expression was erythroid-specific as the transduced myeloid colonies expressed HbF at only 3.4%. In EC, although high background γ-expression was detected in the untransduced samples - probably due to the activated fetal globin synthesis in CD34+cells by the culture conditions -, HbF expression was again higher in the transduced over the untransduced differentiated erythroid cells (58 vs 44%, respectively). Quantitative measurements of γ-globin protein and mRNA from erythroid differentiation cultures as well as integration site analysis in primary and secondary mice, are in process. We here show that the HDAd5/35++ vectors targeted HSCs with high efficiency and may serve as an alternative vector system for gene therapy of hemoglobinopathies over the traditionally used SIN-LVs.

204. Screening Vectors for the Treatment of ß-Globinopathies: a Quest for More Efficient Therapies

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Addition of a functional copy of the ß-globin gene and reactivation of fetal hemoglobin (HbF) are promising therapeutic approaches for ß-globinopathies such as Sickle Cell Disease (SCD) and ß-thalassemia. Results from ongoing clinical trials for ß-globinopathies indicate that a successful outcome is genotype-dependent. In patients with the ß0/ß0 genotype or SCD the vectors using in the trials have failed to produce curative adult hemoglobin (HbA) levels. Therefore, to achieve HbA synthesis at therapeutic levels in the most severe genotypes, and with minimal vector copy number (VCN) per cell, more powerful and versatile vectors are required. Our lab has engineered a new lentiviral vector, ALS10, that carries the ß-globin gene including the non-coding regions. In erythroblasts from patients with SCD and ß-thal the HbA synthesis induced by ALS10 (VCN=1) is 27% and 69% of the total Hb, respectively. In the present study we aim to improve the efficacy of our gene addition approach. A shRNA^{miR} targeting the transcription factor BCL11A, a known repressor of γ-globin, was incorporated into ALS10. The shRNA^{miR} sequences targeting BCL11A (Guda et. al. Molecular Therapy 2015) were flanked by an optimized backbone termed "miR-E" (Fellmann et. al. Cell Reports 2013) for the purpose of increasing HbF levels through down regulation of BCL11A. With this approach we expect to overcome some of the limitations of the vectors presently in clinical trials by simultaneous 1) production of transgenic HbA; 2) reactivation of endogenous HbF; and 3) decrease in production of endogenous mutant protein. First, we used a CRISPR/Cas9 system to mutagenize the ß-globin gene in the HUDEP-2 cell line (Kurita et. al. Plos One 2013) and establish a clonal cell line named M#9, which produces a new hemoglobin variant (Hb-mutant). The ALS10-induced HbA and shRNA^{miR}-mediated HbF are both distinguishable from the Hb-mutant endogenously produced by M#9 via high-performance liquid chromatography (HPLC). Therefore, we can readily assess the production of functional HbA+F hemoglobins after gene transfer and correlate these values to VCN in a dose/effect relationship. Upon transduction of M#9, ALS10 induced 18%, 23% and 44% of HbA at VCN=0.6, 1.0 and 2.0, respectively. We then cloned the miR-E-BCL11A sequence either in the ß-globin intron 1 (ATM1), or in two different regions of intron 2 (ATM2.1 and ATM2.2). Upon transduction of M#9, we observed that ATM1, the best of the ATM vectors, showed production of HbA+HbF equivalent to only ~70% of the total HbA produced by ALS10 (VCN=1). This data suggested that inclusion of the miRNA was not optimal, and lessens production of HbA. To overcome this limitation, we further modified the miR-E-BCL11A in intron 1, generating a vector named ATM1S. With this modification, the production of therapeutic Hbs (HbA+HbF) generated was ~20% superior compared to the amount of HbA produced after transduction with ALS10. Western blot analyses confirmed a concurrent reduction of BCL11A and increase of γ-globin protein levels in samples treated with ATM1S. We are also investigating if ATM1S leads to higher production of therapeutic hemoglobins while avoiding the overproduction of ß-like globin chains (ß + γ + ß^s) when compared to ß-globin-only vectors. Additional studies are in progress using ATM1S, ALS10 and miR-E-BCL11A-only expressing vectors in erythroblasts from patients with SCD. Moreover, we are combining the miR-E-BCL11A of ATM1S with more powerful ß-globin based vectors. In conclusion, our results show that HbA and HbF can be elevated simultaneously using a single lentiviral construct whose ability to induce functional hemoglobin production (HbF + HbA) surpasses that of a vector expressing only ß-globin.

205. Potent HbF Induction Following ssODN-Mediated Repair of Cas9-Induced DSB at the HBG Promoter in CD34+ HSPC

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Sickle cell disease (SCD) is caused by a mutation in the beta-globin gene. Sickle hemoglobin (HbS) polymerizes into fibers that deform red blood cells causing hemolytic anemia and vaso-occlusion. In addition to provoking acute pain, the ischemia caused by vaso-occlusive crises can lead to a large variety of comorbidities, severely reducing life expectancy. Hereditary persistence of fetal hemoglobin (HPFH) is a condition where SCD patients are asymptomatic due to coinheritance of mutations within the beta-globin locus that de-repress the expression of fetal hemoglobin (HbF). HbF inhibits HbS

polymerization, and a sustained expression of high levels of HbF during adulthood prevents the appearance of SCD symptoms. HPFH mutations at the HBG1/2 promoters are clustered in subdomains upstream of the transcription start sites, likely overlapping with repressive regulatory elements. Endonuclease with cut sites overlapping those domains have been used to introduce mutations mimicking the HPFH genotype in hematopoietic stem and progenitor cells (HSPCs) with the aim to provide a curative treatment for SCD. The repair of Cas9-induced double-strand breaks (DSBs) in the HBG1/2 promoter regions often results in small insertions and deletions (indels) of various length and position. Depending on how they affect the binding of repressive factors, the various indels introduced by Cas9 may differ in their capacity to induce HbF expression. We performed in-depth genotype to phenotype analysis in erythroid progeny following delivery of Cas9/guide RNA ribonucleoprotein (RNP) complex targeting the HBG promoter in CD34+ HSPC. Several mutations associated with elevated levels of gamma-globin protein were identified. This analysis guided the rational design of single-stranded oligonucleotide donor templates (ssODNs) that generate high HbF-inducing indels. The dose, length, and symmetry of the homology arms of the ssODN templates were also empirically tested to obtain the highest level of precise repair without affecting cell viability. Consequently, significant increases in HbF expression were detected in the erythroid progeny of mPB CD34+ HSPC treated with RNP co-delivered with ssODN as compared to HSPC treated with RNP alone. In summary, we report a novel approach to promote the directed repair of CRISPR/Cas9 induced DSB in CD34+ HSPC toward mutations associated with potent HbF induction. Engraftment studies in humanized mouse model will determine whether the ssODN-mediated directed repair can occur efficiently in long term hematopoietic stem cell resulting in long-term expression of therapeutically relevant levels of HbF.

206. *BCL11A* Enhancer Disruption by Cas9 RNP Editing of Hematopoietic Stem Cells Ameliorates β-Thalassemia Pathophysiology

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The β-thalassemias are a group of inherited disorders of red blood cells characterized by globin chain imbalance due to underproduction of β-globin. Despite the curative potential of allogeneic bone marrow transplantation, limited donor availability and immune incompatibility remain major barriers to cure. Gene therapies using a patient's own hematopoietic stem cells (HSCs) are a promising approach. Gene addition faces the challenge of high level expression of globin genes and inherent risks of insertional mutagenesis. Gene repair is complicated by hundreds of unique β-thalassemia mutations potentially necessitating a multitude of repair strategies. Here we investigate gene disruption intended to reactivate fetal hemoglobin (HbF) as a potentially universal therapeutic gene editing strategy for the β-hemoglobinopathies including the β-thalassemias. BCL11A is the major repressor of adult expression of fetal γ-globin. The erythroid enhancer of *BCL11A* is required for its expression in adult red blood cell precursors. Natural genetic variation at this enhancer is associated with the clinical severity of β-thalassemia. We identified highly efficient conditions for *BCL11A* enhancer editing by electroporation of Cas9:sgRNA ribonucleoprotein (RNP) complexes into CD34+ hematopoietic stem and progenitor cells (HSPCs), obtaining ~90% indel frequencies. We applied this gene editing strategy to primary CD34+ HSPCs isolated from the nonmobilized peripheral blood of patients with β-thalassemia. We subjected the edited cells to in vitro erythroid liquid culture. We examined HSPCs from patients with $\beta^0\beta^0$, $\beta^+\beta^0$, and $\beta^E\beta^0$ genotypes. In each instance, we demonstrated potent disruption of *BCL11A* expression, induction of γ-globin and HbF, and normalization of β-like to α-like globin ratio. By using flow cytometry, microscopy and ImageStream analysis, we found that edited β-thalassemia HSPCs yielded reticulocytes with increased cell size and more round and uniform morphology as compared to the microcytic hypochromic poikilocytic unedited cells from β-thalassemia patients. These data encourage the clinical development of *BCL11A* enhancer editing as a therapeutic strategy for β-thalassemia.

207. Leukocyte Adhesion Deficiency I: a Closer Step to a Gene Therapy Clinical Trial

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Leukocyte Adhesion Deficiency Type I (LAD-I) is a primary immunodeficiency characterized by recurrent and life-threatening bacterial infections. It is caused by mutations in the *ITGB2* gene, encoding the integrin β_2 common subunit (CD18). These mutations lead to a defective or absent expression of β_2 integrins on the leukocytes' surfaces, rendering leukocytes unable to adhere to the endothelium and extravasate to infection sites. As it is the case with other monogenic immunodeficiencies, LAD-I is a disorder that could be substantially corrected by *ex vivo* gene therapy. We have previously developed a lentiviral vector (LV) in which the expression of the CD18 protein is driven by a Chimeric promoter that resulted from the fusion of the *FES* and the *CTSG* genes minimal 5′-flanking regions. This promoter drives a preferential expression in myeloid cells and has proven its efficacy to correct the LAD-I phenotype both in murine and human LAD-I cells. This LV has recently obtained the Orphan Drug Designation by the EMA (EU/3/16/1753) and FDA (DRU-2016-5430) Agencies. A complete preclinical evaluation of the vector has been carried out including biodistribution, toxicology and safety studies in mice transplanted with Chim.hCD18-transduced Lin- BM cells. These studies demonstrated that the presence of the therapeutic

provirus was restricted to the hematopoietic tissue. Transplantation of mice with LV-transduced or mock-transduced cells did not result in evident alterations in multiple non-hematopoietic organs nor were changes in hematopoietic reconstitution observed. Moreover, none of the gene therapy treated mice showed symptoms of leukemia or insertion site-related clonal expansions. We have also tested the efficacy of the pre-GMP produced Chim.hCD18 therapeutic vectors to transduce CD34+ cells under optimized conventional conditions. In vitro analyses showed the phenotypic correction of LAD-I like hCD34+ cells and the absence of any phenotypic modification of healthy hCD34$^+$ cells after transduction with the therapeutic vector. Moreover, transplantation experiments in NSG mice provided evidence of unaltered engraftment and function of HD HSCs transduced with the therapeutic Chim.CD18-LV. Taken together, these results demonstrate the preclinical efficacy and safety profiles of a gene therapy approach for LAD-I patients.

208. Can "Trisomy Silencing" Correct Known Cell Pathologies of Down Syndrome?

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Previously our lab showed that RNA from an XIST gene inserted into one chromosome 21 in Down Syndrome patient-derived iPS cells could transcriptionally silence genes across the autosome, in cis, reducing Chr21 transcriptional output to near disomic levels. Our lab is working to extend this approach for translational research and potential therapeutic strategies for Down Syndrome, in both human iPS cells and a DS mouse model. A major effort has been to test whether "trisomy silencing" can correct not only transcriptional levels, but known cellular pathologies of Down Syndrome. This was investigated for the hematopoietic system for which DS cell pathologies are well established. Overproduction of hematopoietic cells in fetal liver predisposes patients to myeloproliferative disorder and acute megakaryocytic leukemia. Our results demonstrate that DS associated over-proliferation of megakaryocytes and erythrocytes can be corrected by XIST-mediated chromosome 21 silencing during hematopoietic differentiation of DS iPSCs. Additionally, analysis of early stages of hematopoietic differentiation indicates that trisomy 21 enhances formation of hematopoietic progenitors from hemogenic endothelium. Results further suggest involvement of IGF signaling in trisomy 21 associated over-production of early hematopoietic progenitors. This demonstrates *in vitro* the value of this approach to study pathogenesis of DS phenotypes, but also provides proof-of-principle for the possibility that "trisomy silencing" could be developed as a therapeutic strategy. Our lab is also working on testing *in vivo* correction in human neural cells. Finally, we will describe our progress to test trisomy silencing in the Ts65Dn mouse model by targeted insertion of Xist transgene into the extra trisomic chromosome.

209. Murine T-Cells Can be Efficiently Transduced with Lentivirus Vectors Using a Non-Toxic Enhancer

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Lentiviral vectors (LVs) have emerged as an efficient and safer therapeutic tool not only for hematopoietic stem cell-based but also T-cell based gene therapy trials. However, monitoring of transduced T-cells in appropriate pre-clinical models remains challenging because of inefficient transduction of murine primary T-cells with LVs. In some studies, gammaretroviral vectors have been used to express the transgene in murine T-cells and demonstrate the proof of concept in mouse models. However this pre-clinical strategy is not clinically relevant and there is an urgent need to develop a protocol for efficient transduction of murine cells with LVs. Herein, we describe an optimized gene transfer protocol utilizing a non-toxic lentivirus transduction enhancer to transduce primary murine T cells. The results revealed that our optimised protocol exhibited, for the first time with LVs, the dual benefits of low toxicity and a high efficiency compared to classical protocols. High level transduction of murine CD4$^+$ (60%) and CD8$^+$ (40%) T-cells with VSV-G pseudotyped LV was achieved with no changes in naive or memory phenotypes compared to non-transduced cells. Moreover the transduced murine T-cells demonstrated high stability and survival over the time in *ex-vivo* culture. Thus, this new transduction enhancer overcomes the limitation of using LVs in pre-clinical monitoring of transduced murine T-cells, and will facilitate the translation of such strategies from the bench to clinic.

210. Bio-Distribution and Expression of Valoctogene Roxaparvovec, an AAV5-Based Construct of B-Domain Deleted FVIII, in Mouse Liver

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Hemophilia A (HA) is a bleeding disorder caused by deficiency of circulating clotting factor VIII (FVIII). Valoctocogene roxaparvovec (BMN 270) is an Adeno-Associated Virus 5 (AAV5)-based vector that expresses a B-domain-deleted human FVIII (hFVIII-SQ) under the control of hybrid liver specific promoter (HLP), which is currently being evaluated in severe HA patients. In order for HA gene therapy to be successful, the vector must reach its targeted tissues, be taken-up, the DNA processed and expressed, the FVIII protein folded properly and ultimately secreted. This study aimed to investigate the bio-distribution and persistence of AAV5 capsid protein along with the distribution of vector DNA and hFVIII-SQ protein expression in mouse liver. In addition, two promoters driving the expression of hFVIII-SQ, the HLP and a stronger hepatocyte-specific promoter 100ATGB, were compared to determine the effect of promoter strength on the

percentage of hepatocytes expressing detectable levels of hFVIII-SQ protein. Immunohistochemistry to detect AAV5 capsid protein, VP3, was employed to study AAV5 distribution and persistence in the liver. AAV5 VP3 capsid protein showed a distinct pattern of distribution with the majority of the signal being cytoplasmic, surrounding the central veins of the hepatic lobule. The kinetics of AAV5 capsid clearance was evaluated at 24 hour, 1, 3, 5 and 8 weeks post vector administration. The highest levels of VP3 protein were detected 1 and 3 weeks post-dose. By 5 weeks, the levels of VP3 protein were significantly decreased, and by 8 weeks, there was no detectable VP3 protein in the mouse liver. Similarly, using in situ hybridization techniques, we showed that the hFVIII-SQ transgene preferentially distributed to the hepatocytes in the peri-central region of the liver lobule. Furthermore, there was a dose-dependent increase in the number of hepatocytes that stained positive for the transgene. At 6e13 vg/kg, a dose being tested in the clinic, 93% of mouse hepatocytes stained positive for hFVIII-SQ DNA. To evaluate protein expression and compare HLP and 100ATGB promoters, immunohistochemistry was used to detect hFVIII-SQ protein. Similar to both the VP3 capsid protein and hFVIII-SQ transgene, a peri-central pattern of hFVIII-SQ protein expression was observed. At 6e13 vg/kg, approximately 12.3 ± 3.64 % of hepatocytes in the HLP group stained positive for hFVIII-SQ protein compared to 17.4 ± 4.6% in the 100ATGB group (P >0.05). In conclusion, AAV5 capsid, hFVIII-SQ DNA and B-domain-deleted FVIII protein all showed a similar pattern of distribution with the majority of signal surrounding the central veins. At doses that resulted in therapeutic levels of plasma hFVIII-SQ protein, the majority of hepatocytes stained positive for transgene delivery, however, a lower percentage of the hepatocytes had detectable levels of hFVIII-SQ protein. Lastly, the use of a stronger promoter increased hFVIII-SQ expression in the same subset of peri-central hepaptocytes but did not significantly increase the percentage of cells expressing the protein. Therefore, it is possible that the use of a stronger promoter could lower the vector dose needed to achieve clinically meaningful levels of FVIII, however, this could potentially lead to a cellular stress response as a result of increasing the burden on the same cells to produce, fold, and secrete hFVIII-SQ, a protein known to be difficult to secrete. To date, no evidence of liver dysfunction or cellular stress was observed in mice treated with valoctocogene roxaparvovec that uses the HLP promoter at doses that produced normal to supra-physiological levels of hFVIII-SQ.

211. Improvements in the Transduction Conditions of Human Hematopoietic Progenitors with the CPcoRPKW-17 Therapeutic Lentiviral Vector to be Used in a Pyruvate Kinase Deficiency Gene Therapy Clinical Trial

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Pyruvate kinase deficiency (PKD) is the most common erythroid inherited enzymatic defect causing chronic nonspherocytic hemolytic anemia. PKD is an autosomal recessive disorder caused by mutations in the PKLR gene, which lead to a total or partial reduction of the activity of RPK protein. PKD is associated with reticulocytosis, splenomegaly and hepatic iron overload, and may be life-threatening in severely affected patients. Allogeneic bone marrow transplant has been curative in selected severely affected patients, although it is not considered a standard of care. Lentiviral gene therapy of autologous hematopoietic stem cells may enable restored RPK functionality and could represent a potentially curative, single-treatment approach to PKD. The theoretical clinical benefits of PKD gene therapy were shown previously in preclinical studies conducted in PKD mice, demonstrating the safety and the efficacy of a this new therapeutic lentiviral vector (CPcoRPKW-17) that has been granted orphan drug designation (EU/3/14/1330; FDA #DRU-2016-5168). In order to develop a gene therapy clinical trial for PKD patients, we have developed an optimized transduction procedure compatible with a clinical application by using a GMP-grade lentiviral vector produced according to manufacturing processes of the CMO VIVEbioTECH (www.vivebiotech.com). These viral batches have been tested for transduction efficiency in healthy cryopreserved cord blood and mobilized peripheral blood CD34+ cells comparing different viral concentrations, number of transduction rounds and the use of transduction enhancers. As expected, increased viral doses revealed increasing levels of transduction that ranged from 40-90% in hematopoietic progenitors. Analysis of vector copy number by qPCR ranged from 0.5 to 3 VCN/cell, demonstrating transduction efficiency appropriate for a clinical application. Interestingly, the presence of transduction enhancers conferred an up to 6-fold increase in both the percentage of transduced cells and VCN/cell in a reproducible manner without affecting cell viability, engraftment capacity or differentiation potential of the transduced human hematopoietic progenitors in NSG mice. Altogether, these results demonstrate optimization of the transduction conditions to levels enabling initiation of a forthcoming PKD Gene Therapy Clinical Trial.

212. Inhibition of HIV-1 Infection by Gene Editing of CCR5 and CXCR4 Using CRISPR/Cas9

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The CRISPR/Cas9 system is a transformative genome-editing tool for inducing site-specific genome modifications in human cells. However, translation of this technology for treating human diseases, such as HIV/AIDS, demands exquisite on-target precision and ample efficiency. In this project, we investigated the CRISPR/Cas9 system for creating HIV-resistance, by targeting the human CCR5 and CXCR4 genes, which encode cellular co-receptors required for HIV-1 infection. First, we showed that a single round transduction of lentiviral vectors expressing Cas9 and CCR5 or CXCR4 sgRNAs into CCR5-expressing T-lymphocytes cells yielded significant knockdown of co-receptor expression on the cells. High efficiency of gene disruption of CCR5 and CXCR4 were observed using the Surveyor assay and confirmed by Illumina HiSeq deep sequencing. Gene-disrupted cells were resistant to HIV-1 infection by CCR5 (R5)-tropic, CXCR4 (X4)-tropic, and dual (R5/X4)-tropic strains. Next, we analyzed the differentiation of

CRISPR-derived knockout of CCR5 in human CD34+ hematopoietic stem cells (HSPCs) isolated from cord blood. Colony forming unit (CFU) assays were performed to evaluate the effects of CRISPR-mediated CCR5 disruption on HSPC differentiation. Gene-modified HSPCs were further differentiated into macrophages, which frequently express CCR5 and are thus susceptible to R5-tropic HIV infection. However, upon viral challenge, the CRISPR-modified HSPC-derived macrophages exhibited significant protection from R5-tropic HIV-1 infection. Continuing work on this project includes the analysis of CRISPR-mediated disruption of CCR5 in human CD34+ HSPCs or of both CCR5 and CXCR4 in human PBMCs by ex vivo electroporation of Cas9 nuclease and synthetic crRNA:tracrRNA components. Both experimental strategies involve transplantation of the gene-modified cells in versions of the humanized NSG mouse model (hu-PBL) followed by HIV-1 challenge. These experiments lay the groundwork for advancing this technology as a clinical therapeutic for HIV-1 and other human diseases.

213. High Resolution Longitudinal Monitoring of Hematopoietic Stem Cell Transplantation Using the NIS Reporter Gene and PET/CT

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Bone marrow transplantation (BMT) is used extensively for a broad spectrum of hematopoietic diseases. Longitudinal in vivo tracking of HSC engraftment and regeneration using immunohistochemical staining or analysis of genetically tagged cells in the peripheral blood is suboptimal. While bioluminescence imaging is highly sensitive, it lacks resolution and is not feasible for deep tissues. NIS, the sodium iodide symporter, is a reporter gene that concentrates a variety of radiotracers, making it very convenient to use in small and large animal imaging studies to track virus, gene and cell therapies. Our goal is to use NIS tagged cells to longitudinally monitor sites of HSC homing and engraftment in living animals to better understand hematopoiesis. Lethally irradiated B6SJL mice were transplanted with NIS expressing Sca1+ HSC from C57BL/6, and PET/CT imaging was performed at regular intervals with F18-tetrafluoroborate. Engrafted NIS positive HSC cells could be seen on spine, pelvis and femurs as early as 2 weeks post transplantation. We are currently determining the number of cells needed to identify a single NIS positive focus on the PET image and generate a 3D print of the location of the transplanted cells to better understand preferred homing sites in adoptive cell transfer studies.

Immunological Aspects of Gene and Cell Therapy

214. Busulfan Combined with Immunosuppression Allows Efficient Engraftment of Gene-Modified Cells in a Rhesus Macaque Model

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Busulfan (Bu) conditioning is utilized for hematopoietic stem cell (HSC) gene therapy; however, Bu results in myelosuppression and HSC depletion, but has less impact on adaptive immunity. Therefore, we hypothesized that additional immunosuppression is required for efficient engraftment of gene-modified cells. In this study, we optimized Bu conditioning to achieve efficient engraftment along with immunological tolerance to transgenes in a rhesus HSC lentiviral gene therapy model. At first, Bu pharmacokinetics were performed in 4 animals to target an ablative AUC, and 5.5mg/kg/day for 4 consecutive days (day -4 to -1) was selected. In 2 animals (DEMK and DETL), we transduced half of mobilized $CD34^+$ cells with a GFP-enocoding vector predicted to be immunogenic, and the other half with a γ-globin (GG)-encoding vector without predicted immunogenecity. In both animals, efficient transduction was observed in cultured $CD34^+$ cells (vector copy numbers (VCNs) 1-4 for GFP and 2-3 for GG). The transduced cells were transplanted into autologous animals following Bu conditioning. DEMK demonstrated slow recovery of blood counts, and DETL never adequately engrafted (euthanasia, day 74). We observed transient high %GFP (~90%) among only granulocytes for 1-2 months in both animals, reminiscent of a rejection pattern (Gene Ther. 2014). After 3 months, GFP marking was no longer detectable by flow cytometry in any lineage. Fetal hemoglobin-producing RBCs (F-cells) continued to be detected (~6%) after 6 months in DEMK. Similar VCNs were observed for GFP and GG initially, and GG VCNs stabilized; however, GFP VCNs decreased to 100-fold lower levels by 3 months. GFP antibody production was observed at 3 months in both animals. These data demonstrate that the ablative Bu conditioning alone is sufficient for engraftment of GG-transduced cells, but insufficient to induce immunological tolerance to GFP. The loss of sustained grafts in DETL and poor engraftment in DEMK suggest immunological damage to bystander HSCs. Next, we added immunosuppression with abatacept (20mg/kg x 10, day-1 to 186) and sirolimus (0.025mg/kg, day-14 to 180) to the Bu conditioning. In 2 animals (ZJ50and ZJ32), efficient transduction of $CD34^+$ cells *in vitro* was achieved (VCNs 2-6 for GFP and 8-10 for GG), and earlier robust recovery of blood counts occurred after transplantation. %GFP marking was stable and multi-lineage (3-11%), and VCNs (0.07-0.27 for GFP and 0.004-0.08 for GG) similarly remained stable in both animals through longest followup to date of 6 months. F-cells were detected at 4-6% levels out to 6 months. No anti-GFP antibodies were detectable in either

animal at 3 months. These data demonstrate that the Bu, abatacept, and sirolimus combination allows for engraftment of gene-modified cells, even expressing GFP (immunogenic), presumably due to development of immunological tolerance. In summary, a myeloablative Bu alone resulted in immunological rejection of HSCs transduced with GFP, and bystander damage to non-GFP-transduced cells, while additional immunosuppression with abatacept and sirolimus allowed for engraftment of HSCs expressing either GFP or GG, without an immunoresponse to GFP. This conditioning is more relevant for preclinical development using the macaque model compared to total body irradiation. Bu alone may not allow for efficient engraftment of HSCs expressing a neoantigen, for instance in patients that are protein-null for the target gene prior to gene therapy, and abatacept and sirolimus conditioning should be useful in these conditions.

215. In Vivo Characterization of Exogenous Antibody Secretion by Hematopoietic Cells

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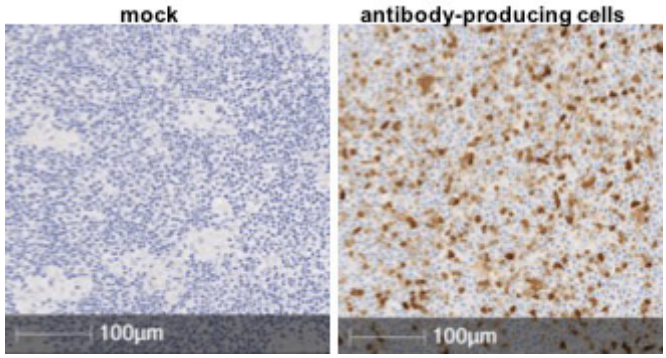
Secretion of therapeutics using gene-modified hematopoietic stem and progenitor cells (HSPCs) presents several advantages for the treatment of chronic diseases. In addition to their indefinite proliferation potential, HSPCs differentiate into various lineages with the ability to cross physiological and anatomical barriers. As such, persistent and local delivery of a therapeutic of interest could be achieved. We sought to assess antibody secretion and delivery using gene-modified HSPCs for the treatment of human immunodeficiency virus, type I (HIV-1). Recent identification of potent broadly neutralizing antibodies (bNAbs) has renewed the possibilities to control virus replication. Several clinical trials have established the safety and promising potential of passively administered antibodies, but the therapeutic efficacy has been limited by their half-lives. Additionally, the existence of latently infected cells in isolated tissue sanctuaries compromises the development of a cure. We evaluated whether antibody secretion by gene-modified HSPCs could address these challenges. Exogenous antibody secretion by $CD34^+$ human HSPCs was assessed *in vitro* and *in vivo* following lentivirus transduction. HSPCs, monocytes and T lymphocytes secreted bNAbs *in vitro*. Stable and persistent antibody secretion averaging 100ng/ml was detected in the peripheral blood of humanized NOD-SCID-gamma mice for up to eight months, and was positively associated with the presence of human $CD45^+$ cells. Engraftment, persistence and differentiation of the human hematopoietic cells were similar between bNAb-producing and non-producing mice. bNAbs mRNA were detected in T and B cells. Additionally, bNAb-producing cells were found in lymphoid tissues such as the spleen, lymph nodes or gut-associated lymphoid tissue at necropsy (Figure 1). The ability of the secreted bNAbs to neutralize the virus *in vivo* is under evaluation. The mice have been challenged with HIV-1 at 20 weeks, when antibody secretion reaches a plateau, and the viremia is being tracked to determine the impact of the secreted antibodies on HIV infection and replication. Our data strongly support that HSPC-based delivery of antibodies will facilitate local delivery of functional proteins to the tissues of interest, in particular potential HIV reservoir sites. More efficient delivery of secreted antibodies to reservoir tissues via our HSPC-based approach may lower effective plasma concentrations relative to those required for efficacy in passive intravenous administration trials. Additionally, administration of factors such as G-CSF or the choice of the conditioning regimen could improve the success rate of this therapy by increasing the trafficking of the gene-modified cells. Future development will focus on optimizing and controlling antibody expression, as well as characterizing the contribution of each hematopoietic lineage to the secretion for a better understanding of the therapeutic potential of this approach. Importantly, this strategy could also be applied to a variety of chronic infectious or non-infectious diseases whose antibodies have been clinically validated, and requiring multiple injections for sustainable efficiency.

Figure 1: GFP immunohistochemistry detection in the antibody-producing gene-modified cells from the mesenteric lymph node of a humanized mice 34 weeks post-infusion.



216. Impacts of Pre-Existing Antibodies on Transduction Efficiency of Systemic AAV9 Gene Delivery: Potential for Broader Patient Eligibility for AAV Gene Therapy

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As AAV gene therapies advance through clinical trials, pre-existing antibodies (Abs) pose a critical challenge for widespread application. To date, for the majority of FDA approved rAAV gene therapy clinical trials, enrollment criteria included either <1:10 αAAV-NAb or <1:100 total αAAV-IgG. The present study assessed the clinically relevant impacts of pre-existing αAAV9-Abs on the transduction efficiency of systemically delivered rAAV9 vector in 2yr-old Cynomolgus macaques that had varying serum levels of αAAV9-IgG: **Group 1:** 1:800-1,600, n=3; **Group 2:** 1:100-400, n=4; **Group 3:** <1:50, n=2. We treated each animal with an IV injection of 1e13vg/kg rAAV9 vector, and tissues were analyzed at 4-week post-injection. The vector treatment increased the transgene expression to above endogenous levels in the brain and all 7 tested somatic tissues in all animals. However, expression level increases were significantly lower (2.9-6.4-fold, $p<0.05$) in virtually all tested tissues in **Group 1** animals compared to **Group 2** and **Group 3**. Importantly, qPCR analyses showed >100-fold lower vector genome (vg) copies in the livers in **Group 1** animals than in **Group 2** and **Group**

3, while no measurable differences in vg levels in the brain, or other tested tissues, were observed among animals in all 3 cohorts. Notably, there were no detectable differences in tissue transgene expression and vg levels between **Group 2** and **Group 3** animals. These data further support our previous findings that moderate levels of pre-existing αAAV9-Abs predominantly diminish transduction in the liver, with relatively little impact on the CNS and other tissues. More importantly, we demonstrate here that pre-existing αAAV9-IgG at≤1:400 (ELISA titers) may have no, or very limited, effects on the transduction efficiency of systemically delivered AAV9 vector. Therefore, our data here support the possibility of raising the bar to use ≤1:400 pre-existing αAAV-IgG as an enrollment criteria in future AAV gene therapy clinical trials, which may broaden patient eligibility for AAV-mediated gene therapy treatments.

217. Exploring Protein Orthogonality in Immune Space: A Case Study with AAV and Cas9 Orthologs

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A major hurdle in protein-based therapeutics is the interaction with the adaptive immune system, which can lead to neutralization by circulating antibodies and clearance of treated cells by cytotoxic T-lymphocytes. One method of circumventing these issues is to use human or humanized proteins which avoid the immune response by self-recognition. However, this approach limits potential protein therapeutics to those of human origin, excluding many exciting effectors and delivery vehicles such as CRISPR-Cas9 and adeno-associated viruses (AAVs). To address this issue, we propose here the sequential use of orthologous proteins whose function is constrained by natural selection, but whose structure is subject to diversification by genetic drift. This would, in principle, allow for repeated treatments by 'immune orthogonal' orthologs without reduced efficacy due to lack of immune cross-reactivity among the proteins. To explore and validate this concept we chose 91 Type II CRISPR-Cas9 orthologs and 167 AAV capsid protein orthologs, and developed a pipeline to compare total sequence similarity as well as predicted binding to class I and class II Major Histocompatibility Complex (MHC) proteins. Interestingly, MHC binding predictions revealed wide diversity among the set of Cas9 orthologs, with 83% of pairs predicted to have non cross-reacting immune responses, while no global immune orthogonality among AAV serotypes was observed. To confirm these findings we selected two Cas9 orthologs, from *S. pyogenes* and *S. aureus*, predicted to be orthogonal in immune space, and delivered them into mice via multiple AAV serotypes. We observed cross-reacting antibodies against AAV but not Cas9 orthologs in sera from immunized mice, validating the computationally predicted immune orthogonality among these proteins. Moving forward, we anticipate this framework can be applied to prescribe sequential regimens of immune orthogonal protein therapeutics to circumvent pre-existing or induced immunity, and eventually, to rationally engineer immune orthogonality among protein orthologs.

218. Anti-Inflammatory Cytokine Encoding Lentivirus-Mediated Gene Therapy Enhances Functional Regeneration Following Spinal Cord Injury

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Introduction Initial traumatic spinal cord injury (SCI) causes a primary injury, with the secondary inflammatory responses resulting in permanent loss of functions. Herein, we investigated nerve regeneration after SCI following local immunomodulation using lentiviral vector delivery of anti-inflammatory cytokines (IL-10 or IL-4) from a multichannel bridge. IL-10 and IL-4 were investigated because of their dual neuroprotective and neuroregenerative abilities. We hypothesized that the architectural aspects of the bridge would synergize with the immunomodulation provided by cytokines to modulate neuroinflammation and promote regeneration. **Methods** Lentiviral vectors loaded-multichannel bridge was implanted into the lateral hemisection SCI mouse model (Fig.1). The inflammatory response was characterized histologically, and through transcriptome analysis. The number of axons and their extent of myelination were characterized histologically, with locomotor tests performed for functional recovery. **Results** The localized expression of IL-10 or IL-4 were able to attenuate the neuro-inflammatory microenvironment as determined through microarray analysis, and furthermore, this expression of anti-inflammatory factors induced a cascade of expression for gene associated with neural regeneration and neural development. Interestingly, the mechanical guidance provided by the multichannel bridge synergized with the anti-inflammatory cytokines to recruit cells into the site while polarizing them towards pro-regenerative phenotypes, induce axonal regrowth and remyelination, and improve functional recovery after SCI (Fig.2). **Conclusion** These results suggest that providing a permissive environment for regeneration by a multichannel bridge, combined with localized anti-inflammatory cytokine gene delivery may provide a novel therapeutic strategy to overcome multiple barriers to modulate an inhibitory neuro-microenvironment, and enhance functional recovery and regeneration after SCI.

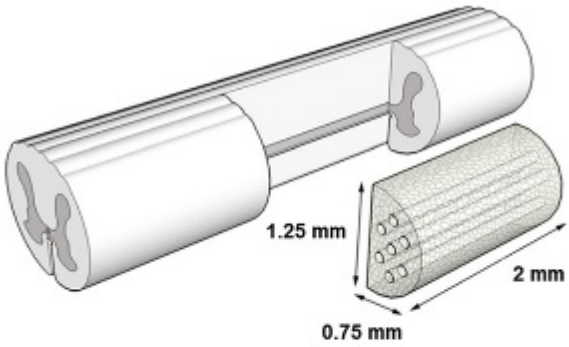

Fig.1. Schematic representation of hemisection SCI at T9-T10 and bridge implantation in the injury site

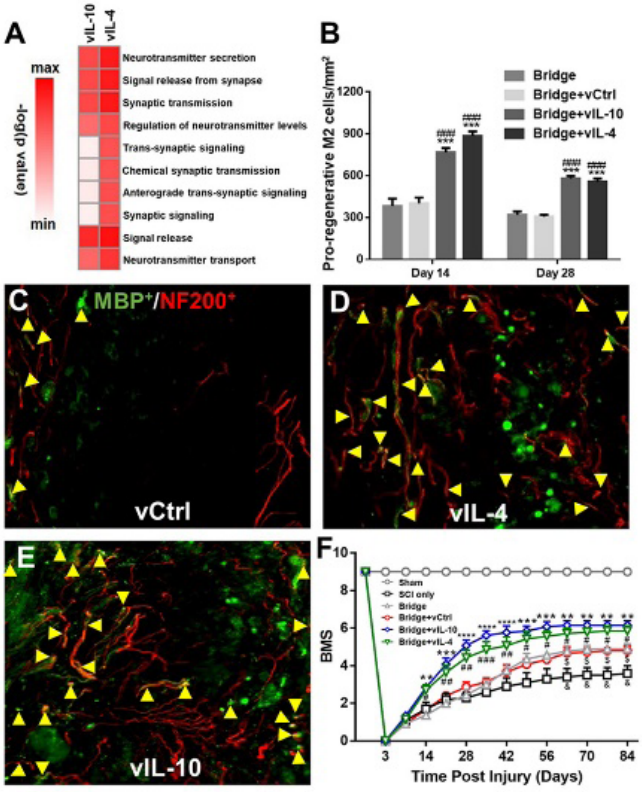

Fig.2. (A) Upregulated ten gene ontologies by IL-10 and IL-4. (B) The density of M2 macrophage by IL-10 and IL-4. Myelinated axons within the bridge delivering (C) vCtrl, (D) vIL-4, and (E) vIL-10. (F) Recovery of locomotor function after SCI by anti-inflammatory cytokines

219. Assessing Anti-Dystrophin T-Cell Responses by Elispot Following AAV9-Microdystrophin Gene Therapy in Dogs

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Duchenne muscular dystrophy (DMD), caused by mutations in the X-linked dystrophin gene, affects approximately one in 3,500 to 5,000 newborn males, making it the most common terminal genetic childhood disease. It is differentiated from the milder Becker muscular dystrophy by a complete or near-complete absence of the dystrophin protein, often the result of unstable mRNA due to early stop codons and frame-shifting mutations. A recent focus of treatment for DMD is the use of adeno-associated virus (AAV) mediated gene transfer to induce expression of a shortened form of dystrophin (microdystrophin) in muscle to reduce disease severity. In order to assess anti-transgene immunological responses, we conducted a pre-clinical assessment of T-cell responses to a novel microdystrophin AAV9 vector cassette, which utilizes the muscle-specific promoter CK8, in DMD dog colonies at two different sites. The first colony is the golden retriever-derived model (GRMD or CXMD), with a point mutation at the 3′ splice site of intron 6 resulting in skipping of exon 7, a frame shift and an early stop codon in exon 8. The second colony is a mixed mutation colony, with the GRMD mutation, the Pembroke Welsh corgi insertion of LINE-1 in intron 13 resulting in a novel exon with an early stop codon, and the Labrador retriever insertion of 184 base pairs in intron 19, resulting in a nonsense mutation. Dogs received doses between 1e13 and 5e14 vg/kg, or vehicle control, delivered systemically at 2-4 months of age with transient immune suppression and were followed for at least 3 months. All treated dogs showed positive dystrophin expression, and dogs assayed by ELISpot were negative for interferon gamma T-cell response at all timepoints (baseline, 1 and 3 months post administration for colony 1; baseline, 1.5 and 6 months post for colony 2). All vaccinated dogs showed positive T-cell responses to the DAPPv vaccine, suggesting it serves as a good choice for a physiologic positive control. Given that dogs have been shown to be good models for transgene-directed immune responses following gene therapy in other diseases, this multi-site canine study supports the hypothesis that systemic gene therapy trials using AAV and a muscle-specific promoter to drive microdystrophin expression may prove to be safe as well as efficacious.

220. TLR7 Activation Fails to Enhance Antibody Formation against Factor IX in Muscle Gene Transfer

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Innate immune signals that promote B cell responses in gene transfer are not well defined. A recent study in our laboratory showed that co-administration of AAV1 expressing human factor IX (AAV1-hFIX) and the TLR9 agonist ODN-1826 (class B CpG ODN) induced antibody formation against hFIX by two weeks (*Cell. Immunol.*, in press). This effect correlated with activation of monocyte-derived dendritic cells (moDCs) and enhanced T follicular helper (Tfh) cell responses. Interestingly, a less robust but still measurable increase in anti-hFIX formation, resulting in a reduction of systemic expression, was still observed in $TLR9^{-/-}$ mice. This observation led us to hypothesize that, while TLR9 is the major determinant of ODN-1826's effect on antibody formation, other TLR9-independent pattern recognition receptors (PRRs) play a role as well. A study by others showed that $MyD88^{-/-}$ or $TLR7^{-/-}TLR9^{-/-}$ in C57BL6/J mice results in ablation of innate cytokine production in response to murine cytomegalovirus (MCMV) infection, a double-stranded DNA virus (*J. Immunol.*, 180:9, 5799). Further analysis of the responses in mice deficient in TLR7 or TLR9 suggested that CpG ODNs to a lesser extent may also activate TLR7 (like TLR9, TLR7 is also located in endosomes, but primarily recognizes RNA molecules). TLR7 signaling is also known to activate memory B cells, making it a potential candidate for promoting antibody responses. In our present study, we therefore further investigated the possibility of TLR7 activation augmenting antibody formation following AAV gene transfer. C57BL6/J mice (n=8) received intramuscular injections of either AAV1-hFIX and resiquimod (TLR7/TLR8 agonist) or AAV1-hFIX and CL264 (TLR7-specific agonist) using vector doses of $1x10^{11}$ vg/mouse. Control mice (n=8) received AAV1-hFIX alone. Mice were monitored for at least ten weeks for circulating hFIX levels and anti-hFIX IgG formation. While hFIX-specific antibodies formed in all groups, there were no significant differences compared to control mice at two weeks for the resiquimod group (mean=107.7 ng/mL, SD=66.8; control mean=475.8 ng/mL, SD=493.8) or the CL264 group (mean=1142.2 ng/mL, SD=1493.1; control mean=985.7 ng/mL, SD=1180.5) or at any other time point and hFIX expression persisted in plasma. These results suggest that activation of TLR7 does not contribute to antibody response against transgene products expressed from an AAV vector in skeletal muscle. The sum of our published and unpublished data indicates that specific TLR9 agonists but not agonists of TLR2, 3, 4, and 7 induce B cell responses to muscle-derived secreted transgene products. Furthermore, TLR9 sensing of the AAV genome is not required but merely has a modulating role and fails to have a measurable effect on moDC or Tfh responses. Thus, only specific innate receptors activated by certain types of ligands promote antibody formation in muscle gene transfer. Presently, we are in the process of acquiring flow cytometry data from mice injected with either AAV1-hFIX alone or in combination with different TLR7/8/9 agonists (resiquimod, CL264, ODN-1826). These data will provide in-depth knowledge about the frequencies and activation status of different cell types following administration of these TLR7-activating adjuvants.

221. Induction of Immune Tolerance towards a Human Protein- HexM Expressed through AAV Gene Transfer in Sandhoff Mice

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Sandhoff disease (SD) and Tay-Sachs disease (TSD) are neurodegenerative disorders caused by the toxic accumulation of GM_2 ganglioside in the brain. This is due to a deficient enzyme, beta-hexosaminidase A (HexA), responsible for the breakdown of this ganglioside. HexA is a heterodimer consisting of αλπηα and βετα subunits; a defective βετα subunit results in SD and a defective αλπηα-subunit, TSD. A recently constructed enzyme analogous to HexA, called HexM, can efficiently catabolize GM_2 gangliosides, while its gene, *HEXM*, can be compactly packaged into the self-complementary adeno-associated virus 9 (scAAV9) vector. Gene transfer studies have revealed that the scAAV9-*HEXM* treatment in SD mice results in significant survival benefit. However, our recent mouse study also showed that scAAV9-*HEXM* has the potential to provoke an immune response against the AAV capsid and the expressed human HexM product. This undesirable immune response can prove to be an obstacle for the long-term efficacy of the treatment, especially in individuals who have no native HexA protein (e.g., in infantile forms of the diseases). We hypothesized that suppressing the immune system will allow the body to tolerize the treatment and newly produced HexM protein. Rapamycin (R) and prednisone (P) are the immuno-suppressants (IS) chosen due to the regulatory T-cell sparing effect, and both are clinically used drugs to suppress cytotoxic immune responses (cellular against AAV capsid and humoral against HexM). Using an SD mouse model ($hexb^{-/-}$) we administered scAAV9-*HEXM* and used R & P to diminish the immune response to the treatment. Six-week old mice were injected intravenously with scAAV9-*HEXM* with or without IS (singly or combined) for short- (R- 4 weeks and/or P-2 weeks + taper over 5 weeks) or long-term (R - 13 weeks and/or P- 11 weeks + taper over 5 weeks) regimen. IS was administered daily starting at 5 weeks, and serum was collected at 4 week intervals. Mice were monitored monthly for survival and behaviour. This study had 3 endpoints: 3 weeks post-gene therapy treatment, 10 weeks post-treatment, and at the humane endpoint. Organs, serum, and splenocytes were obtained at the endpoints for further analysis. Humoral and cellular immune responses to the HexM therapeutic protein were tested in addition to assays for hexosaminidase enzyme activity and transgene copy number analysis in different tissues. Preliminary data have revealed that administering R (long-term) with P (long-term or short-term) with scAAV9-*HEXM* showed a reduction in antibody and IFN-g T cell responses to HexM (reduction continued after ISs were stopped), increased hex enzyme

activity levels in serum and increased the survival benefit as compared to mice receiving scAAV9-*HEXM* without IS. Further copy number analysis, histology and assays of brain Hex levels are on-going. The outcomes of this study may provide a proof-of-concept tolerization IS regimen for long-term efficacy of not only the scAAV9-*HEXM* treatment, but also other gene therapy studies using AAV. This study may prove to be a major stepping-stone towards clinical gene therapy for the treatment of SD and TSD.

222. Broad Antibody Cross Reactions to Different AAV Serotypes in NHPs Following an Intravenous AAV9 or AAV1 Vector Delivery

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While different AAV serotypes are broadly defined as serologically distinct, it is becoming clear that many antibodies cross-react between them. As AAV gene therapies advance through clinical trials, pre-existing antibodies (Abs) pose a critical challenge for translation with respect to patient eligibility and potential need for re-administration. In this study, we assessed antibody responses to 9 AAV serotypes (AAV1-3, 5-9 and rh74) in non-human primates (NHPs), 4-weeks following an IV injection of 1e13vg/kg rAAV9 (n=7) or rAAV1 (n=5) vector, to determine potential alternatives to AAV9 or AAV1 as gene delivery vectors. Intravenous injection of AAV9 led to significant increases in total IgG (by ELISA) to all tested serotypes in 6 animals that had relatively low (1:50-1:400, n=4) or high (1:800-1,600, n=3) levels of pre-existing αAAV9-Abs. In contrast, one NHP that had pre-existing αAAV9-Abs at 1:50 prior to AAV9 administration showed low Ab responses to 8 of the tested AAV serotypes, with the exception of AAV6. The AAV1 treatment in NHPs with no (n=3) or low (1:100) pre-existing α-AAV1-Abs led to Ab responses to 8 of 9 tested AAV serotypes, but not to AAV5, in four animals. In the remaining animal, low level Ab responses to AAV1, 2, 3, 8 and Rh74 (1:50-200) were observed, with no cross-reactivity to AAV5-7 or 9. These data further support the notion of broad Ab cross-reactivity across AAV serotypes. Therefore, alternative natural AAV serotypes may not be feasible as vectors for initial AAV gene delivery in individuals with pre-existing αAAV-Abs in general, or for re-administration. Effective Ab depletion will still be the desired strategy to overcome pre-existing αAAV-Abs for AAV-mediated gene delivery in humans.

223. Tolerogenic Nanoparticles Enhance Transgene Expression after Both First and Second Administrations of AAV Gene Therapy Vectors through Immunological and Non-Immunological Mechanisms

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Achieving clinically active and stable expression of therapeutic transgenes is a key challenge for gene therapy. In particular, transgene expression following liver-directed systemic gene therapy in pediatric patients is expected to wane over time due to vector dilution with growth. The formation of neutralizing antibodies prevents the ability to re-administer AAV. We have previously shown that tolerogenic nanoparticles encapsulating rapamycin (SVP-Rapamycin) have the ability to enable transgene expression following a second administration of AAV by blocking humoral and cellular responses against the capsid (Meliani et al., ASGCT 2017). Here we further demonstrate that co-administration of SVP-Rapamycin with AAV-based vectors leads to dose-dependent and long-term suppression of humoral and cellular responses against AAV, thereby enabling transgene expression to be boosted following repeat administration. Moreover, co-administration of SVP-Rapamycin enhances transgene expression even following the first dose of AAV vector in naïve mice. This enhanced expression is immediate, dose-dependent and is not a function of regimen or the route of administration. Moreover, this first dose enhancement of transgene expression does not appear to be directly related to suppression of adaptive immunity, but is likely linked to rapamycin-mediated induction of autophagy, which was recently reported to increase AAV transduction and transgene expression (Hősel et al, Hepatology 2017). This two-pronged mechanism of SEL-110 action in vivo makes it an attractive candidate to enhance systemic gene therapeutic applications, particularly in those clinical indications where repeat vector dosing may be necessary.

224. Definition of a Combination Therapy for Modulating Immune Responses after In Vivo Hepatocyte-Directed Lentiviral Vector-Mediated Gene Transfer

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Liver-directed lentiviral vector (LV)-mediated gene transfer is becoming a concrete therapeutic opportunity for the definitive correction of several monogenic diseases. However, the systemic injection of LV triggers transient innate inflammatory response favoring the induction of anti-transgene adaptive immune responses, which may result in transgene clearance. This is the case of alpha-L-iduronidase (IDUA) KO mice, the murine model of Mucopolysaccharidosis type-I (MPS-I) in which in vivo hepatocyte-directed IDUA gene transfer failed in restoring enzymatic activity due to immune-mediated clearance of IDUA-expressing hepatocytes. Using MPS-I murine model, we are testing combined therapies, to uncouple IDUA presentation and innate immune signals after LV gene therapy to favor IDUA-specific tolerance. The selective depletion of T cells by anti-CD3 F(ab')2 tolerogenic regimen only partially modulated anti-IDUA responses, without affecting innate response. Therefore, to define potential targets of a combination therapy to favor tolerance induction upon hepatocyte-restricted transgene expression, we investigated kinetics of innate response-related cytokines and chemokines in the serum of both mice and large animals after LV administration. Beside the well characterized type-1 interferons response induced upon viral genome sensing by plasmacytoid dendritic cells (DC), we found significantly increased levels of several pro-inflammatory cytokines (IL-1b, IL-5, IL-

6, IL-18) and chemokines (MCP-1, MIP-1a, MIP-1b, Rantes, KC) 3hrs post LV administration. Moreover, in vivo blockade of NLRP3 pathway by pharmacological inhibitors resulted in normalization of IL-1b and IL-18 production, indicating that inflammasome activation occurs after systemic LV injection. Short glucocorticoid treatment, which inhibited up-regulation of pro-inflammatory cytokines and chemokines except IL-1b, did not improve immune-regulation induced by anti-CD3 regimen. Conversely, when IL-1 signaling was selectively blocked by IL-1 Receptor Antagonist (IL-1RA), IL-1b, IL-18, IL-5, MCP1 serum levels were normalized, and anti-CD3-mediated regulation of anti-IDUA responses was further improved. These data indicate that combination therapies targeting innate and adaptive responses may improve the efficacy of in vivo gene therapy in MPS mice. Additional strategies are under investigation to identify translatable combination of biologicals capable to synergize with hepatocyte-directed gene transfer in establishing IDUA-specific tolerance. Results will further expand the safety and applicability of LV in vivo gene therapy directed to hepatocytes for monogenic diseases.

225. Prevalence of Neutralizing Antibodies against Adeno-Associated Virus Serotypes 1, 2, and 9 in Non-Injected Latin American Patients with Heart Failure

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Introduction: Heart failure is a common, costly, and potentially fatal condition that affects about 40 million people worldwide. Available therapies alleviate patients' symptoms without reversing the pathological process. In this context, gene therapy emerges as an alternative for a curative solution. The activity of neutralizing antibodies (NAbs) against the viral capsid of recombinant adeno-associated viral (rAAV) vectors, however, is a hurdle that decreases the efficiency of vector transduction thus limiting the expression of the transgene. Interestingly, NAbs prevalence varies depending on gender, age, serotype, and mainly, geographic location. Furthermore, cross-reactivity (antibodies against one serotype recognizing capsid residues from other serotypes), has also been reported. To date, there are no data on the prevalence of NAb in Latin American population, hindering the implementation of rAAV vector-based clinical trials in this population. Here, we sought to determine the prevalence of NAbs against different rAAV vectors (serotypes 1, 2, and 9) in patient-derived serum from individuals with heart failure in Colombia. **Methods**: Serum was collected from 57 participants that were seen at the outpatient Heart Failure Clinic at Fundación Cardiovascular de Colombia (FCV). NAb were detected using an *in vitro* inhibitory assay. HEK 293 cells were plated on a 96-well plate at $2x10^4$ density and then infected with an rAAV-dsCB-GFP vector that was previously incubated with dilutions of each participant's serum. The plate was evaluated 24 hours after the infection. The neutralizing titer of the sample was reported as the first dilution at which ≥50% inhibition of the GFP signal was achieved. A neutralizing titer of >1:50 was considered positive (*per Food and Drug Administration cut-off point for AAV-clinical trials).* **Results**: Participants characteristics: 25% women, average age 60.5 years (range: 20-87, SD: 14.1 years). 56.1% of the samples were positive for NAbs for at least one serotype. The most prevalent serotype in the population was AAV2 (46.6%), followed by AAV1 (33.3%), and AAV9 (21%). Interestingly, 25% of the samples presented NAbs for two or more of the serotypes analyzed and 34.3% of the positive samples were positive for NAbs against the three serotypes. Almost all participants that were positive for AAV1 (73.7%), and AAV9 (91.6%), were also positive for AAV2. **Conclusions:** This sample of Colombian heart failure patients have higher levels of AAV2 and AAV1 exposure compared to previous reports from the USA, but lower than reports from Africa and Asia. Though AAV2 levels are higher, AAV1 levels in this study are lower than reported in European countries, suggesting that AAV1 might be endogenous to the European zone. AAV9 NAbs' levels in this study do not present a significant difference with previous reports worldwide. Importantly >33% of the positive samples were simultaneously positive for all serotypes analyzed, which might suggest coinfection or, likely, a high level of cross reactivity of the NAbs among serotypes. These results provide evidence, for the first time, of AAV natural exposure in Latin American individuals, which is an important consideration for the design of future rAAV research in the region. A second phase of the study will assess healthy individuals and the possible role of socioeconomic variables in the prevalence of Nab.

226. Insulin as a Pharmaceutical Agent to Blunt Innate Immune Responses to AAV Vectors

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The results of this study demonstrated that a short term insulin treatment significantly reduced gene expression of pro-inflammatory cytokines in *in vitro* stimulation models using human and mouse white blood cells (WBCs). We previously demonstrated that a short term insulin therapy co-administered with AAV vector delivery significantly improves transduction of liver and skeletal muscle *in vitro* and *in vivo* (Carrig et al., HGT 2016). Following these results, our lab investigated if insulin could be used as a pharmaceutical agent to limit innate immunity against AAV vectors. First, we established the *in vitro* model in cultured WBCs using TLR2 and TLR9 agonists separately and combined for synergy. We used mouse splenocytes and a differentiated human monocyte cell line (U937). We measured gene expression of IL1b, IL6, IL12, IFNy, TNFa, TLR2 and TLR9. The U937 cells were differentiated with 10uM of PMA for 24hrs followed by 48hr recovery then challenged with agonists; mouse splenocytes were cultured for 48hrs before stimulation. First we established the optimal stimulation times and dose response curve for the agonists. Both 2hr and 6hr stimulation times with 500nM of TLR2 agonist and/or 1uM of TLR9 agonists showed the highest increase in cytokine gene expression in splenocytes and differentiated U937 cells. For all studies combining TLR2 and TLR9 agonists had the highest degree of

gene expression and undifferentiated U937 did not respond as well to agonist challenge. To verify differentiation of U937 cells, we measured a 9 fold increase in cd11b. We also assessed insulin receptor (*INSR*) gene expression using comparative quantitative PCR. We previously showed the human lung A549 cell line had the lowest degree of *INSR* expression while differentiated human myocytes had the highest (14 Fold increase compared to A549) (Carrig et al., HGT 2016). We saw similar results in mouse tissue biopsies. For this study mouse splenocytes showed a 4 fold increase in *INSR* expression compared to lung tissue and a 2.5 fold decrease compared to skeletal muscle indicating that, while expression of *INSR* is lower than skeletal muscle, splenocytes can respond to insulin therapy. We saw similar results when comparing the *INSR* expression in the differentiated U937 cells to lung A549 cells and differentiated myocytes. Next, we co-administered 5 ug/ml of insulin with the agonists which significantly reduced gene expression of pro-inflammatory cytokines in both stimulated and unstimulated cells at both 2hr and 6hr exposure times (see table). We then tested the stimulation model with a self-complimentary (sc) AAV vector. scAAV vector alone showed a trend of increasing gene expression of pro-inflammatory cytokines but this increase was not significant. Therefore we co-administered the vector with a 10 fold lower dose of TLR9 (100nM) which significantly increased the pro-inflammatory cytokines (see table). A 100nM TLR9 treatment alone did not result in a significant increase in gene expression. Again, when we co-administered insulin with the scAAV vector and the low dose of TLR9 agonist, there was a significant decrease in gene expression (see table). These data clearly demonstrate that insulin is a pharmaceutical candidate to blunt innate immune responses against AAV vectors.

Table 1: Fold Change Compared to Unstimulated Cells

Cell Type	Treatment	IL1β	IL6	IL12	IFNγ	TNFα	TLR2	TLR9
Spleen	TLR2+TLR9-2hr	9.7	4.8	23.2	2.4	4.2	2.2	3.4
Spleen	TLR2+TLR9+Ins-2hr	2.1	1.4	5.8	0.91	1.2	0.89	0.72
Spleen	TLR9+scAAV-2hr	6.7	5.3	14.6	3.8	2.4	1.9	3.9
Spleen	TLR9+scAAV+Ins-2hr	1.3	0.84	4.2	0.93	1.1	0.79	0.63
U937	TLR2+TLR9-2hr	3.2	6.5	9.8	5.8	3.8	2.5	2.8
U938	TLR2+TLR9+Ins-2hr	0.95	1.2	1.6	0.57	0.81	0.91	1.1
U939	TLR9+scAAV-2hr	2.4	7.8	8.7	3.1	2.6	1.7	4.6
U940	TLR9+scAAV+Ins-2hr	0.82	1.4	1.8	0.73	1.2	0.88	0.79
Spleen	Only Ins-2hr	1.1	0.82	0.52	0.83	1.3	1.1	0.89
U937	Only Ins-2hr	0.67	0.34	0.78	0.31	0.71	0.58	0.65

227. Mitigating T Cell Response to AAV Vectors for Gene Therapy through Engineered Red Blood Cells

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Gene therapy delivery via adeno-associated virus (AAV) vectors has shown promising early results in a range of disorders including coagulation disorders, inherited blindness, and neurodegenerative diseases. However, challenges related to immune-mediated rejection of the AAV vector and transduced cells limit therapy efficacy for some patient populations. A majority of people have detectable neutralizing antibody titers and memory T cells specific for AAV vector proteins due to prior exposure to AAV. When there is pre-existing immunity to AAV, immune mediated clearance of the vector or transduced cells limits therapeutic efficacy of the initial dose and precludes administration of additional doses. While the reaction to AAV can be mitigated with steroid-based immune suppression or certain patient populations can be excluded, immune reaction is a clear and critical hurdle for AAV-based gene therapy. Induction of AAV vector-specific immune cell tolerance could reduce unwanted immune reactions, enhance gene therapy efficacy, and enable administration of multiple doses of AAV-based gene therapies. At SQZ Biotech, we are developing a tolerogenic approach that utilizes red blood cells (RBCs) loaded with AAV-derived antigens to mitigate the AAV-specific immune response. Recent research has found that antigens associated with eryptotic RBCs are processed in the spleen in a tolerogenic manner. To exploit the tolerogenic process of eryptosis, we delivered AAV-specific antigens to RBCs using the microfluidic CellSqueeze® technology. In this technology, cells undergo temporary permeation by passing through constricting channels. The applied force destabilizes the cell membrane, enabling the diffusion of material into the cell for a short time before the membrane reseals. Previous work has shown the CellSqueeze® technology has high delivery efficacy in patient-derived cells, such as stem cells and immune cells, and with a variety of target molecules. We also demonstrated efficient delivery of cargo into human and murine RBCs. Building on these initial results, we developed a murine model of AAV-specific T cell memory to test whether we can induce tolerance to AAV vectors. We used CellSqueeze® to deliver AAV derived peptides to mouse RBCs and adoptively transferred these RBCs to AAV-reactive mice. Treatment with loaded RBCs resulted in deletion of AAV-specific T cells and blunted effector response of remaining virus specific T cells upon restimulation. Applying the CellSqueeze® technology to RBCs enables development of an allogeneic cell therapy which deletes virus-specific T cells. With this capability, we have developed a novel, safe, efficient method to deliver gene therapy associated antigens to promote tolerance and overcome a key barrier to the broad clinical application of gene therapy.

228. Combination of an Engineered AAV Vector Anc80 and Tolerogenic Nanoparticles Encapsulating Rapamycin Enables Efficient Transgene Expression in Mice with Neutralizing Antibodies to Natural AAV Serotypes

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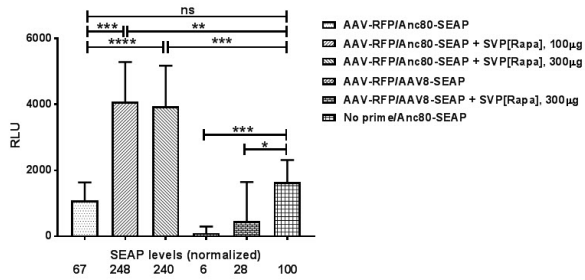
Two immunological barriers to gene therapy using adeno-associated virus (AAV)-based vectors stem from the high prevalence of pre-existing neutralizing antibodies (Nabs) from natural AAV infections in the human population, which may completely prevent transduction by therapeutic AAV vectors, and the inability to re-dose patients due to *de novo* formation of Nabs induced by the initial administration of AAV. We have developed tolerogenic nanoparticles encapsulating rapamycin (SVP-Rapamycin) which have been shown to effectively block the formation of anti-drug antibodies when co-administered

with a variety of biologic drugs. SVP-Rapamycin has been successively used to block Nab formation when co-administered with a therapeutic enzyme, pegsiticase, for the treatment of severe gout and is now being evaluated in phase 2 clinical trial. We have previously shown that co-administration of SVP-Rapamycin with AAV-based vectors leads to dose-dependent and long-term suppression of humoral and T cell responses against AAV and therefore allows for productive re-dosing of AAV vectors. Here we demonstrate that SVP-Rapamycin can enhance AAV transduction in the presence of low levels of pre-existing antibodies against common AAV8 serotype. Although, the benefit of SVP-Rapamycin is limited in the presence of high titers of pre-existing antibodies, this impediment may be circumvented by utilizing Anc80, a rationally engineered AAV vector. Anc80 vector expressing secreted embryonic alkaline phosphatase (SEAP) combined with SVP-Rapamycin enabled efficient transgene expression in mice that were previously exposed to AAV8 and developed AAV8 Nabs. The level of SEAP expression was similar to that observed in naïve mice administered Anc80-SEAP vector alone (Fig. 1). In addition, SVP-Rapamycin significantly increased transgene expression when co-administered with an Anc80 vector, but not AAV8 vector, in mice receiving passive transfer of sera from human donors with pre-existing antibodies to AAV. Moreover, co-administration of Anc80 and SEL-110 suppressed *de novo* generation of antibodies against Anc80 in a dose-dependent manner thus enabling its repeat administration. The combination of SVP-Rapamycin with an Anc80 vector is promising approach to mitigate the detrimental impact of both pre-existing and *de novo* formed neutralizing antibodies on gene therapy. Fig. 1. Anc80-directed transgene expression in AAV8-immune mice is augmented by SVP[Rapa]. Mice (6-9 per group) were immunized with 1×10^{11} vg/kg of AAV8-RFP followed by injection of 5×10^{11} vg/kg AAV8-SEAP or Anc80-SEAP ± 100 or 300 µg SVP[Rapa] on d57. SEAP expression measured on d64.



229. Characterization of Human Pre-Existing Cellular Immunity against Aav 2, 4, 5, 8 and 9 Serotypes

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Recombinant adeno-associated virus (rAAV) is the most used viral vector for *in vivo* gene therapy. Pre-existing T cell responses to AAV capsid can hamper rAAV-mediated gene transfer efficiency and safety in patients. To our knowledge, few studies have reported the prevalence of AAV-specific T cell responses in the general population, particularly against AAV 8 and 9 serotypes that have recently emerged as promising tools for clinical applications. Furthermore, functional read-outs that could be correlated to clinical outcome in patients remain to be found. In our study, we used IFNγ ELISpot assays to investigate the prevalence of circulating T lymphocytes directed against AAV 2, 4, 5, 8, and 9 serotypes among healthy human donors. Interestingly, we evidenced that responses directed against AAV9 were the most prevalent with nearly 50% of positive donors. Using CD4+ and CD8+ T cell magnetic depletions, we found that positive responses were predominantly mediated by CD8+ T cells. To further investigate the functionality of anti-capsid T cells, fluorospot-based assays assessing simultaneously IFNγ, TNFα and IL2 secretions were used. T cell functionality appeared dependent on AAV serotypes. Finally, our data confirmed a high prevalence of cross-reactive responses in humans. Overall, characterizing anti-AAV cellular responses in a multiparametric and serotype-dependent way should prove to be the way to improve clinical pre-screening and immune monitoring of patients in AAV-based gene therapy clinical trials.

230. Gene Therapy Vectors Expressing Immunomodulatory Proteins from Diverse Viruses Inhibit T Cell Response *In Vitro*

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Introduction: Host immune responses to gene therapy viral vectors and its components limit therapeutic efficacy and present a safety risk. Novel strategies are necessary to lower immunogenicity in order to advance gene-transfer therapies. Diverse human viruses have evolved to evade host immune response and cause infection. Identifying and characterizing viral factors that evade the immune response may provide additional tools against host immune evasion of viral vectors. Here we characterized viral proteins from five distinct human viruses for their ability to inhibit human T cell response in vitro. **Methods**: Using bioinformatics immunomodulatory viral proteins were identified for their predicted ability to inhibit human T cell function. Inhibition of human T cell function by predicted viral proteins or short motifs were measured in human T cell lines in vitro. Gene therapy vectors (lentiviral or gamma-retroviral) were generated to express viral immunomodulatory proteins, and T cell response was assessed by transducing human T cells and measuring T cell activation. **Results:** Group based prediction system (GPS) identified five viral immunomodulatory proteins or motifs from five distinct human viruses (HBV-HBsAg, HCV-NS5A, Zika-NS5, YFV-Env and HTLV-I gp21E). Expression of HBV-HBsAg, HCV-NS5A, and YFV-Env but not Zika-NS5 inhibited human T cell function. Lentiviral vector or gamma-retroviral vector expressing viral proteins or short-motifs significantly inhibited human T cell response in vitro. **Conclusions:** These data demonstrate that GPS can be useful to identify viral immunomodulatory proteins; though GPS is not perfect and requires further optimization. Incorporation of characterized immunomodulatory proteins or motifs in the design of gene therapy vectors may be an effective strategy to reduce vector immunogenicity *in vivo*.

231. Superior Immune Modulation and Anti-Arthritic Activity by Cybrocell™ Compared to Bone Marrow Mesenchymal Stem Cells

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Introduction: Regenerative cells have demonstrated a variety of anti-inflammatory activities, of which in some cases, translate to ability to modulate autoimmune processes. The current study was performed to compare immune modulatory activity of bone marrow mesenchymal stem cells (BM-MSC) and CybroCell fibroblast derived cells. **Methods**: The ability of BM-MSC and CybroCell to evoke immune modulation was assessed by addition to ongoing mixed lymphocyte reaction (MLR). Ability to inhibit T cell proliferation and cytokine production was quantified by thymidine incorporation and ELISA. In vivo therapeutic activities of BM-MSC and CybroCell where assayed by intravenous administration in the collagen II model of rheumatoid arthritis and assessment of clinical score, immunohistochemical assessment, and immune recall responses. **Results:** In MLR assays CybroCell was consistently superior to BM-MSC at inhibiting T cell proliferation and production of pro-arthritic cytokines TNF-alpha, IL-1, IL-6 and IL-17. In the collagen induced arthritis model, both CybroCell and BM-MSC where capable of suppressing clinical score. Higher levels of inhibition were observed in the CybroCell treated groups. Furthermore, immunological assessment of collagen 2 recall response revealed augmented levels of CD4+ CD25+ T regulatory cells in the CybroCell treated group. **Conclusions:** These data suggest that CybroCell possesses immunological activity superior to BM-MSC. It appears that immune modulation is responsible for superior activity of CybroCell in animal models of rheumatoid arthritis in terms inhibiting disease progression.

Metabolic, Storage, Endocrine, Liver and Gastrointestinal Diseases I

232. Bi-Allelic cleavage by Cas9 Causes Translocation between Homologous Chromosomes -Therapeutic Opportunities and Mechanisms in Non-Dividing and Dividing Cells

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Patients suffering from monogenic recessive genetic disorders are often genotyped as compound heterozygous, i.e., two homologous chromosomes carrying two different loss-of-function mutant alleles of a gene. Here, we report a genome-editing strategy that uses no exogenous DNA repair template to recombine the pre-existing, correct genetic information present in the two heterozygous alleles into one functional allele. This is achieved by Cas9-mediated, bi-allelic cleavage at an intronic sequence between the two mutation sites, followed by translocation between the homologous chromosomes. We term this strategy as allelic exchange. Recombinant adeno-associated virus (rAAV)-delivery of Cas9 and a single sgRNA induced inter-allelic repair in a mouse model of hereditary tyrosinemia type I (HT1) that carries compound heterozygous *Fah* gene mutations, rescuing the disease phenotype in neonatal or young adult mice. In contrast, a different sgRNA designed to induce gene conversion was not able to rescue the disease phenotype, suggesting that the inter-allelic repair is predominantly a consequence of translocation between homologous chromosomes, rather than a homology-directed repair (HDR) event. In the heart of a compound heterozygous mouse model of mucopolysaccharidosis type I, we were able to partially correct the biochemical defects by allelic exchange, further suggesting the potential therapeutic applications of allelic exchange in non-dividing cells. Using a fluorescent reporter system and a human cell line deficient in LIG4 (a key player in canonical non-homologous end-joining, cNHEJ), we found that DNA translocation in human cells is dependent on cNHEJ. Because NHEJ is active throughout the cell cycle, we further examined whether allelic exchange can also occur during mitosis in dividing cells. By taking advantage of several dimorphic genetic markers present in the compound heterozygous HT1 mice, we analyzed the segregation patterns of these genetic markers following allelic exchange during mitosis. Our results support that allelic exchange can occur between duplicated chromatids of homologous chromosomes during mitosis. Overall, allelic exchange repair is a versatile therapeutic genome editing approach that targets recessive compound heterozygous mutations prevalently found in patients. (PDZ, WX, GG: co-corresponding authors)

233. Ex Vivo Hematopoietic Stem Cell-Based Gene Therapy for Mucopolysaccharidosis Type I

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Lysosomal storage disorders are prime candidates for ex vivo lentiviral gene therapy using autologous hematopoietic stem and progenitor

cells (HSPC), due to the possibility to overexpress a functional copy of the defective enzyme and biodistribute it systemically with the help of hematopoietic progeny re-installing normal enzyme bioavailability in the tissues. Hurler Syndrome, the severe form of mucopolysaccharidosis type I (MPSI-H), is caused by α-L-Iduronidase (IDUA) deficiency leading to systemic glycosaminoglycan accumulation, skeletal abnormalities, neurodevelopmental decline and death within the first decade. Gene therapy restores IDUA expression (>20x above normal) leading to full correction of disease manifestations in the homologous mouse model. A series of in vivo mouse toxicity studies performed under GLP conditions (n=132 mice) confirm safety and efficacy of the gene therapy approach. An anti-IDUA immune response represents a potential risk of gene therapy in immunized MPS1 subjects (see Abstract by Squeri et al.), to be addressed with a myeloablative and immunosuppressive conditioning regimen in a future clinical trial. We reasoned that a shortened ex vivo transduction protocol could allow HSPC homing before transgene expression has started, i.e. within 14hr post lentivirus (LV) exposure, and sustain faster hematopoietic reconstitution. Using human BM and MPB CD34+ cells, we optimized a novel transduction protocol requiring only 36hr of ex vivo culture. In the context of this protocol, Prostaglandin E2 (PGE2) allowed sustaining transduction efficiency to similar levels as was achieved with a 60-hour protocol and 2 exposures to IDUA LV. Mechanistically, PGE2's effect on HSPC transduction was specific for VSVg-pseudotyped vectors and dependent on EP2 and EP4 receptors, since pharmacologically inhibiting both but not one of the receptors nearly abrogated its activity. Moreover, continuous PGE2 stimulation during LV exposure achieved higher transduction than a 2hr pulse. As shown by 2 GLP studies on healthy donor BM and MPB CD34+ cells transduced with IDUA LV, shortly-manipulated cells engrafted to higher levels in hematopoietic organs and the brain of NSG mice as compared to cells transduced with the 60hr protocol (total of 67 mice), overcoming the negative impact of ex vivo culture and boosting hematopoietic recovery after conditioning. Both the shorter culture time and PGE2 contributed to higher HSPC engraftment. Integration site analysis on SCID-repopulating cells after 24 weeks showed polyclonal engraftment, and no integration site skewing attributable to the new transduction protocol. Importantly, BM CD34+ cells from 3 MPSI-H patients were efficiently transduced with IDUA LV, engrafted NSG mice to high levels and installed supraphysiologic and normal IDUA activity in whole blood and plasma, respectively. We have filed an application for a phase I/II clinical trial enrolling patients affected by MPSI-H.

234. ZFN-Mediated In Vivo Genome Editing Results in Therapeutic Levels of α-Galactosidase A and Effective Substrate Reduction in Fabry Knockout Mice

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Fabry disease (FD), an X-linked lysosomal storage disease, is caused by mutations in the *GLA* gene encoding α-galactosidase A (α-GalA). FD is characterized by progressive systemic accumulation of the enzyme's substrates, globotriaosylceramide (Gb3) and lyso-Gb3, leading to renal, cardiac and/or cerebrovascular disease and culminating in premature demise. The disease is most commonly treated by enzyme replacement therapy (ERT). However, ERT requires a lifetime of biweekly infusions and may not clear all substrate from secondary organs. A more effective and long-lasting treatment would benefit FD patients. Therefore, the effectiveness of a liver-targeted Zinc-Finger Nuclease (ZFN)-mediated genome editing strategy that permanently integrates a therapeutic human *GLA (*h*GLA)* gene in the *Albumin* locus in hepatocytes was evaluated in a knock-out mouse model for Fabry disease (GLAKO mice). This approach ensures long-term expression of the transgene by exploiting the high level transcriptional activity of the native *Albumin* enhancer/promoter in stably modified hepatocytes and utilizes an endogenous promoter, obviating this requirement in the AAV payload. GLAKO mice received a single injection of AAV encoding a h*GLA* cDNA donor in the presence of *Albumin*-targeted ZFNs under the control of a liver-specific promoter. Co-administration of these three AAV vectors achieved up to 250x wild type α-GalA activity in plasma, which was sustained for the 2 month study, and supraphysiological activities in liver, heart, kidney and spleen. Gb3 and lyso-Gb3 concentrations in these tissues decreased to normal levels. To estimate the fraction of hepatocytes that had been successfully modified by our genome editing strategy, liver expression of the Albumin-h*GLA* donor fusion mRNA was measured via in situ hybridization assay at two months post-transduction. The percentage of liver cells expressing the Alb-h*GLA* mRNA ranged from 5.6% (low dose of ZFNs and h*GLA* donor) to 9.8% (high dose). These studies provide "proof-of-concept" for ZFN-mediated genome editing of hepatocytes to express high levels of human α-GalA,s leading to high enzyme activity in plasma and target tissues.

235. CRISPR/Cas9-Mediated Correction of a Murine Model of Hereditary Tyrosinemia Type 1

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Adeno-associated virus (AAV) vector is one of the ideal systems to correct human genetic diseases due to its low immunogenicity, reduced oncogenic risk, and broad range of serotype specificity. Although this system has many advantages, the major obstacle to AAV-mediated gene repair is the low frequency of gene correction *in vivo* due to inefficient homologous recombination. In the current study, we used the CRISPR-associated RNA guided endonuclease Cas9 to promote homologous recombination near the mutation site in hepatocytes in a mouse model of the human disease hereditary tyrosinemia. A two-virus system was employed: one AAV virus carries a SpCas9 expression cassette driven by a small liver specific promoter, and another AAV virus harbors U6 promoter-driven sgRNA and a fragment of *Fah* genomic DNA as the donor for homologous recombination. We used AAV8 to deliver the sgRNA, DNA repair donor, and SpCas9 via intravenous injection. In neonatal mice, gene correction frequencies of 11 to 25% of hepatocytes were achieved. The efficiency in adult mice was significantly lower at

only 0.5%. The discrepancy between neonatal and adult animals could be explained by lack of hepatocyte proliferation in older animals. Homologous recombination at DNA double-strand breaks is known to occur preferentially in the S-phase of the cell cycle. We therefore tested agents that can induce hepatocyte replication in adult animals. Here we demonstrate that administration of the thyroid hormone triiodothyronine (T3) stimulated hepatocyte proliferation and boosted the gene correction efficiency by up to 10-fold. Additional molecules are being tested in combination with T3 to further enhance in vivo gene targeting efficiency.

236. A Novel Hybrid Promoter Directing AAV-Mediated Expression of Acid Alpha-Glucosidase to Liver, Muscle and CNS Yields Optimized Outcomes in a Mouse Model of Pompe Disease

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Pompe disease is caused by mutations in the acid alpha-glucosidase gene (GAA) that is responsible for processing lysosomal glycogen. Patients with Pompe disease exhibit clinical phenotypes across a variety of tissues, including glycogen buildup in cells, deficits in cardiac, respiratory, and skeletal muscle function, and CNS pathology. Enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA) ameliorates some of these deficits; however, its clinical efficacy has been limited by immunogenicity and lack of uptake of rhGAA into some affected tissues. We propose that optimal multi-tissue expression driven by a gene transfer vector may address the deficits that have been refractory to ERT and promote immune tolerance to hGAA. To achieve multi-tissue correction while avoiding potential immune complications induced by ubiquitiously expressing promoters, we designed a series of expression cassettes to direct expression of hGAA to distinct tissues and/or combinations of tissues, and tested their function when delivered via AAV8 or AAV9 in the murine GAA-/- model of Pompe disease. We generated vectors containing the desmin promoter (Des), a liver specific promoter (LP), and several novel hybrid promoters based on the desmin promoter, designed to target expression to muscle, liver, and neural tissues (MLNP1, 2, and 3). A total of 6 rAAV preparations were tested at multiple systemic doses: (1) rAAV8-LP-hGAA; (2) rAAV9-Des-hGAA; (3) rAAV8-MLNP1-hGAA; (4) rAAV8-MLNP2-hGAA; (5) rAAV8-MLNP3-hGAA; and (6) rAAV8-Des-hGAA. Consistent with increasing levels of liver contribution to the MNLP hybrid vectors, we observed elevated liver GAA activity in MLNP1 > MLNP2 > MLNP3 > desmin promoters without loss of GAA activity in muscle for MLNP1 and MLNP3 constructs relative to desmin-only promoters. GAA activity was also elevated in diaphragm, heart, brain and spinal cord in mice dosed with rAAV8-MLNP1-hGAA relative to vehicle-treated control mice. Vector copy numbers across a range of tissues were consistent between vectors of varying promoter or capsid serotype. Moderate to strong expression of hGAA in the liver by vectors 1, 3, and 4 was sufficient to induce humoral tolerance to hGAA in treated mice, indicating that choice of a hybrid promoter could modulate host immunity to transgene. Conversely, weak to absent liver expression in vectors 2, 5, and 6 was associated with development of elevated anti-hGAA antibody levels. Crucially, dosing with rAAV8-MLNP1-hGAA reduced glycogen levels and rescued Pompe-associated pathology in quadriceps to near-WT scores. Finally, hGAA transcripts were observed in spinal cord of mice dosed with rAAV8-MLNP1-hGAA by RNA-seq analysis. Our findings represent the first nonclinical application of an engineered hybrid promoter providing tissue-specific neuromuscular transgene expression combined with immune tolerizing liver expression, and support the clinical translation of Vector 3 as an optimized hGAA vector for AAV gene therapy for Pompe disease.

237. Naked-DNA Minicircle-Vector Gene Transfer to Periportal Hepatocytes Corrects Ureagenesis in Ornithine Transcarbamylase (OTC)-Deficient Spf-Ash Mice

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Liver gene therapy for pediatric patients with non-integrating vectors is challenging due to hepatocellular proliferation during growth. Here, we are exploring episomal, non-viral naked-DNA minicircle-based vectors that can be reapplied for liver-directed gene therapy to target periportal hepatocytes in male spf-ash mice, a model for the X-linked OTC deficiency. OTC deficiency is the common defect in the urea cycle that is active exclusively in periportal hepatocytes and is responsible for removing ammonia from the blood. Targeting minicircle-vectors to periportal hepatocytes requires administration through vessels connecting directly to the portal triad composed of branches of portal vein, hepatic artery and biliary duct. Surgical methods were established that allowed for hydrodynamic vector administration directly into either the portal vein or the biliary tract of a mouse. While portal vein injections were accompanied by high mortality in spf-ash mice (57% lethality, 26/46 mouse died), application via hydrodynamic retrograde intrabiliary infusion (HRII) was much safer with 94% survival (92/98 mice survived) and potentially superior over the portal vein access as passage through fenestrated endothelia is not required. We used luciferase-expressing minicircles to establish delivery conditions with *in vivo* imaging (IVIS) in combination with quantitative immunohistochemical staining for periportal (OTC) and pericentral (glutamine synthetase) hepatocytes. As therapeutic vector, we generated an expression cassette with a codon-optimized mouse *Otc*-cDNA and a truncated intron that is under transcriptional control of a liver-specific promoter-enhancer sequence. In pilot studies, we

treated spf-ash mice based on elevated liver OTC activity (up to 77% of wild-type enzyme activity), normalization of *in vivo* ureagenesis using stable isotopes (reached wild-type levels), and selective survival of severe hyperammonemia induced upon an shRNA-mediated knock-down of residual endogenous OTC expression. While this study proves that non-viral naked DNA/minicircle-vectors correct OTC deficiency in spf-ash mice, the HRII method still needs optimization.

238. Evaluation of Recombinant Adeno-Associated Virus-Based Genome Editing Reagents for Homology-Directed Repair to Target a Human Liver Locus In Vivo

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Momentum surrounding the use of vectors based on adeno-associated virus (AAV) for gene therapy applications in the liver has increased dramatically, with therapeutic efficacy demonstrated in patients with haemophilia A and B. Human hepatocytes are therefore a compelling target for both gene delivery and genome editing technologies. Given that access to patient cells with metabolic disease phenotypes is limited, our group has developed a novel "minigene" system containing an exact copy of the human mutation targeted for repair. Notably, we selected the human *OTC* gene as a clinically relevant target locus and delivered this "minigene" to the liver of newborn, Otc-deficient, *spf^{ash}* mice by intraperitoneal injection of a *piggyBac* transposon/transposase system packaged into rAAV2/8. This resulted in chromosomal integration of the target sequence into the murine hepatocytes. Three weeks later, animals were treated with rAAV2/rh10 vectors, one containing a repair template for homology-directed repair (HDR) and, to enhance rAAV-mediated HDR, a second vector expressing CRISPR/Cas9 and single guide RNA selected based on its superior ability to direct Cas9 to the "minigene". Using this approach, we identified guide strands for effective target site cleavage and observed gene correction of the introduced human OTC mutation in the target cells. This "minigene" system provides a novel approach to functionally validate genome editing reagents, specifically guide strands and rAAV-based HDR templates, which can then be directly adapted for potential human application. We are currently extending the use of these reagents to treat FRG mice engrafted with primary human hepatocytes.

239. Pharmacological Regulation of Transgene Expression via Chemically Induced Dimerization for Treatment of Neuropathic Lysosomal Storage Diseases

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Hematopoietic stem cell transplantation (HSCT) or HSC-mediated gene therapy has emerged as a potent therapeutic approach for the treatment of lysosomal storage diseases (LSDs). However, many obstacles are encountered in the clinical application, including loss of control over genetically engineered cells in recipients and poor penetration of therapeutics to the brain. We have recently demonstrated the feasibility of enhancing therapeutic efficacies by transiently promoting erythropoiesis and thrombopoiesis in multiple organs of MPS I mice by repeated phlebotomy. We reported here a controllable selection approach to increase genetically corrected, transgene-overexpressing cells in multiple blood lineages by using a well-studied small molecule, chemical inducer of dimerization (CID) or AP20187. Hereditary mutation of α-L-iduronidase (IDUA) causes mucopolysaccharidosis type I (MPS I) and/or Hurler Syndrome for the neuronopathic MPS I disease. A lentiviral vector (LV-SF-IDUA.2a.GFP-ires-F36Vmpl) was constructed to co-express three proteins under the spleen focus-forming virus (SF) promoter. It includes human IDUA, GFP and the CID-responsive element F36Vmpl which consists of a CID-specific dimerization site and intracellular c-MPL kinase domain. We tested the hypothesis that CID can trigger the dimerization of the fusion receptor and active c-MPL pathway in multiple lineages using three cells lines. After 21-days of continuous treatment, CID increased LV-transduced human megakaryocytic Dami cells (GFP+ cells) from 20% to 30%, with 2-fold elevation of IDUA activity released into the media. In human monocytic U937 cells, CID selection showed significant GFP% increase from 31% to 42%, with 1.8-fold upregulation of IDUA activity detected in media. Human K562 cells, which can be differentiated into RBC, monocytes and macrophages, demonstrated comparable results. Moreover, to determine if the proliferative response is tightly controlled by CID, cells were exposed to three cycles of 7-day addition and 7-day withdrawal of CID in all three lineage-specific cell lines. The percentage of GFP+ cells gradually increased during each week of CID addition but remained unchanged during the weeks of drug withdrawal. It has been reported in HSCT recipients that myeloid compartments can migrate into brain to surmount the blood-brain barrier in adults, which may serve as potential producers/depots for CNS delivery of therapeutics. We examined reconstitution of donor-derived myeloid cells in the brain after neonatal transplantation of HSCs derived from GFP transgenic mice via busulfan conditioning. FACS analyses showed that donor-derived CD45+CD11b+ repopulated in the myeloid compartment of the recipient brain ~2-months after transplantation, with 20% GFP+ cells found in meningeal and choroid plexus macrophages ($CD11b^{+}CD45^{high}MHC^{high}$). Immunofluorescence staining revealed that the repopulated GFP^{+} cells are mainly distributed at thalamus, brain stem and midbrain. Additional efforts are underway to evaluate in vivo the feasibility of CID-controlled proliferation in

multiple lineages, especially in monocyte/macrophages. Our data demonstrated that CID could stimulate selective proliferation of F36Vmpl-containing cells with increased production and release of IDUA in erythroid, megakaryocytic and monocytic lineages in a controllable and repeatable manner. These findings may open a door for further improvement of therapeutic efficacies in HSC-mediated gene therapy, a repeatable and controllable approach that may benefit multiple organs including the brain.

240. Single Cell RNA-Seq Reveals Tissue Localization and Transcriptional Signatures of Transduced Hepatocytes Isolated from Non-Human Primates Following Treatment with AAV8

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Single cell RNA sequencing has proven to be a powerful technique to characterize the cellular transcriptome with unprecedented, single cell resolution. In our current work, we utilize single cell RNA-seq to study the transcriptional landscape of primary hepatocytes isolated from rhesus macaques following treatment with an AAV8 vector expressing GFP. Transcriptome analysis of FACS-sorted GFP+ and GFP- cells reveals tissue localization of transduced cells within the hepatic lobule as well as genes and regulatory pathways involved in hepatocyte transduction and the regulation of transgene expression. For our study design, rhesus macaques were treated with either 1×10^{13} genome copies (GC)/kg AAV8.TBG.EGFP.WPRE (n=1) or 1×10^{13} GC/kg AAVG3.TBG.EGFP.WPRE (AAV8 variant, n=1). Animals were euthanized 7 days following treatment, and necropsies were performed to isolate the liver from both animals. Following treatment with collagenase and gradient centrifugation, isolated hepatocytes were FACS sorted by GFP transgene expression onto BD Precise™ 96 well plates. 192 single cells were isolated from each animal (96 GFP+ and 96 GFP-) and were subsequently used to prepare single cell RNA-seq libraries following the standard BD Precise™ protocol. Data were analyzed using the Seurat, Scran, and Scater packages in R in order to determine differentially expressed transcripts between GFP- and GFP+ sorted cells and to perform spatial reconstruction of isolated cells within the hepatic lobule using established transcriptional expression signatures. AAVG3 was found to have an increased liver tropism and exhibited a 1.5-fold increase in transduction efficiency as compared to AAV8. Single cell transcriptome analysis of sorted hepatocytes reveals transgene-expressing cells are evenly distributed across the hepatic lobule, showing a slight preference for the periportal region, which was also observed by histopathology. Interestingly, a subpopulation of sorted GFP- cells are found to express the transgene transcript at levels comparable to sorted GFP+ cells, suggesting that these cells are in fact transduced and express transgene mRNA, despite the absence of detectable levels of translated protein. Comparing the transcriptional profiles of GFP- and GFP+ cells reveals differentially expressed transcripts involved in viral mRNA translation, elucidating possible pathways involved in the regulation of transgene protein expression in transduced cells.

241. Correction of Visual and Auditory Function by AAV9-Mediated Gene Therapy in a Mouse Model of Mucopolysaccharidosis Type IIIB

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Mucopolysaccharidosis type IIIB (MPSIIIB) is a rare autosomal recessive lysosomal storage disease caused by a deficiency in the lysosomal enzyme alpha-N-acetylglucosaminidase (NAGLU), involved in the catabolism of the glycosaminoglycan heparan sulfate. Besides progressive brain neurodegeneration and neuroinflammation, and mild somatic alterations, patients also experience severe vision loss and hearing disturbances. In a previous study, we demonstrated the efficacy in correcting both brain and somatic MPSIIIB-associated disease in a mouse model after an intra-cerebrospinal fluid (CSF) administration of an AAV9 vector encoding for the murine NAGLU enzyme. Here, we examined the ability of this gene therapy approach to counteract visual and auditory deficits in aged MPSIIIB mice. To this aim, AAV9 vectors encoding for the NAGLU protein were administered into the CSF of 2-month-old MPSIIIB mice and the therapeutic effect on hearing and visual function was evaluated 4 and 10 months after vector delivery, respectively. Four months after vector administration, MPSIIIB-treated mice showed a preservation of the cochlear cytoarchitecture. Moreover, the auditory-evoked brainstem response (ABR) evidenced a complete recovery of the hearing function in 50% of MPSIIIB-treated mice, and the other 50% showed partial ABR amelioration. At 12 months of age, electroretinography and histopathological evaluation of retinas from MPSIIIB-treated mice showed a complete restoration of cone function and an amelioration of rod-driven responses. This correlated with a significant improvement in visual discrimination learning and acuity in the water maze and optomotor tests. This study demonstrates the improvement of the auditory and visual functions in aged MPSIIIB mice after AAV9-NAGLU gene therapy.

242. Enhanced Gene Delivery to the Kidney by Viral and Non-Viral Vectors

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There are a plethora of genetic and acquired renal diseases that might be addressed by gene therapy. Gene delivery to the kidney from the blood is inefficient in part due to the small (10 nm) diameter of afferent

arterioles that feed the glomerulus. If a vector passes this bottleneck, it then confronts the glomerulus, which generally only passes small (~10 kDa) proteins. Given these issues, we evaluated the ability of popular small adeno-associated virus (AAV) vectors, larger adenovirus (Ad) vectors, and non-viral DNA formulated with polyethyleneimine (PEI) to deliver genes to renal cells. These vectors were delivered by intravenous (IV), retrograde ureteral (retro-ureteral), and kidney subcapsular injections in mice. Levels of transduction were quantified by Xenogen IVIS luciferase imaging and a luciferase enzyme assay of kidney homogenates. These data show that the vectors mediated weak transduction in the kidney after IV injection. In most cases, IV injection mediated transgene expression in the glomerulus and rarely in downstream cells. In contrast, both AAV and Ad mediated substantial transduction in off-target tissues like the liver after IV injection. Retro-ureteral and subcapsular injections into the kidney mediated markedly increased transduction in kidney tubule cells, whereas non-viral PEI vector mediated nearly undetectable transduction by these routes. Ad vectors mediated more rapid and higher expression than either AAV9 or AAVrh10 even when 10-fold higher genome copies of AAV were used relative to Ad. Large Ad vector transduction remained confined to the kidney after retro-ureteral or subcapsular injection. In contrast, AAV9 and AAVrh10 leaked into the systemic compartment after kidney injections and transduced off-target tissues. Retro-ureteral and subcapsular injections of AAV or Ad-Cre marked tubule cells in LoxP-mRFP-LoxP-mGFP mice and LoxP-Stop-LoxP-Luciferase mice. Administration of AAV9 "Brainbow" vectors by retro-ureteral or subcapsular injections generated stochastic marking of tubule cells in Ksp1.3/Cre mice. In most cases, Brainbow marking documented transduction of single cells with single vectors. Only rarely was co-transduction observed by two AAVs. These data suggest that these injection routes are viable for single gene and single vector therapeutic approaches. These data also suggest that genome editing strategies that require co-delivery of more than one vector to one cell (e.g. Cas9 + gRNA + repair template) may have difficulties in delivering all of the needed genetic payloads into the same cell to achieve therapy. While increasing doses of multi-vector systems may overcome this problem, increasing doses may also precipitate dangerous side effects, particularly if the vectors leak into the blood.

243. CRISPR-Enabled Gene Correction of Methylmalonic Acidemia

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Methylmalonic acidemia (MMA) is an autosomal recessive metabolic disorder, most commonly caused by pathogenic variants in the mitochondrial enzyme methylmalonyl-CoA mutase (*MUT*). This enzyme is an intermediate in the pathway that converts isoleucine and valine to succinyl-CoA. Despite advancement in the understanding of disease pathophysiology and resulting improvements in mortality, patients with MMA caused by MUT deficiency continue to exhibit severe morbidity. There are several lines of evidence that suggest partial restoration of MUT activity in the liver may be therapeutic in MMA. Long-term follow-up however has revealed a propensity for mice treated with adeno-associated viral (AAV) gene therapy as neonates to develop hepatocellular carcinoma based on promoter/enhancer configuration of the vector in a dose-dependent fashion. We hypothesize that programmable nucleases such as Cas9 should make it possible to reduce the deleterious effects of off-target integration by mediating correction of the pathogenic variants at the disease locus. AAV-mediated delivery of CRISPR components and a homologous recombination (HR) cassette carrying cDNA for methylmalonyl-CoA mutase in a disease model of MMA will enable correction in sufficient number of hepatocytes to ameliorate the MMA phenotype. We have identified target sites in the *Mut* gene and in the safe harbor locus albumin. The small percentage of cells corrected by HR will result in therapeutic levels of protein expression due to the high transcriptional activity of albumin as well as its liver-specific expression. *In vivo* screening of the *Staphylococcus aureus* Cas9 and guide RNA cassette showed measurable non-homologous end joining activity (20.86% ± 2.809, n=5) at the *Mut* locus and (21.03% ± 0.5457, n=3) at the *Alb* locus. This strategy is highly modular and can be applicable to other inborn errors of metabolism, particularly those for which secretion of the enzyme can lead to cross-correction of diseased hepatocytes.

244. Hepatic Changes Associated with Chronic Alcohol Exposure in an Alpha-1 Antitrypsin PiZ Mouse Model

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The PiZ mutation in the alpha-1 antitrypsin (AAT) gene causes the PiZ mutant protein to be sequestered in the endoplasmic reticulum of hepatocytes, causing significant liver pathology in ~10% of PiZZ homozygous AAT disease patients. Current transgenic mouse models of the disease include the liver-specific over-expression of mutant PiZ protein. However, these animal models do not fully recapitulate the liver disease found in PiZZ homozygous patients. Since only a small percentage of patients develop liver disease and it is not reproducible in animal models of AATD, it suggests that there are other factors that participate in disease pathogenesis. Here, we propose that in the presence of alcohol, liver injury will be initiated and that the intensity of the disease will be exacerbated by the presence of accumulated PiZ mutant protein. To test this hypothesis, we have administered alcohol via the Lieber-DeCarli diet regimen to PiZ transgenic and control C57Bl/6 mice for 12 weeks. We found that chronic alcohol exposure induced hepatocyte damage in PiZ mice to a greater degree than that seen in alcohol fed wild-type C57Bl6 mice when compared to their respective PiZ and C57Bl6 control-diet cohorts, as determined by rises in serum AST. We also observed increased fibrosis, steatosis and inflammation in the livers of alcohol fed PiZ mice over those of control alcohol fed mice. The fibrotic changes were the most marked. These findings are consistent with a chronic low-level hepatic insult seen in chronic alcohol consumption. We then treated PiZ mice at post-

natal day 1 with a promoter-less adeno-associated (AAV) vector that expressed wild-type AAT as well as a synthetic miRNA to silence the endogenous PiZ allele. We evaluated the liver pathology and AST levels in both alcohol and control diet fed mice following gene correction.

245. Development of *In-Vivo* Oxidation of 1-^{13}C-Propionate as a Surrogate Endpoint for Clinical Outcomes in Propionic Acidemia

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Background: Propionic acidemia (PA) is an inborn error of organic acid metabolism caused by pathogenic variants in *PCCA* and *PCCB* and results in the impaired function of propionyl-CoA carboxylase. PA is characterized by accumulation of propionic acid metabolites in body tissues and is associated with multi-organ pathology including failure to thrive, cardiomyopathy, stokes, metabolic instability, renal dysfunction, bone marrow suppression, and impairment of sensory and central nervous systems. To date, our ability to predict clinically relevant outcomes based on genotype is lacking. We hypothesized that *in-vivo* 1-^{13}C-propionate oxidation represents a proxy biomarker for enzyme activity that could closely correlate with severity of pathogenic variants, biochemical parameters, growth, clinical outcomes, and cognitive functioning scales. *Methods*: We evaluated 18 patients at the NIH Clinical Center enrolled in "The Natural History, Physiology, Microbiome and Biochemistry Studies of Propionic Acidemia" protocol (ClinicalTrials.gov Identifier: NCT02890342). To assess the residual activity of propionyl-CoA carboxylase, we administered sodium 1-^{13}C-propionate as a single oral or gastric bolus. Total carbon dioxide in breath samples was collected serially for two hours using disposable breath collection kits. Baseline VCO_2 production (ml/min) was measured by indirect calorimetry. Isotopomer enrichment in the expired CO_2 ($^{13}CO_3/^{12}CO_2$ ratio) was analyzed using isotopomer ratio mass spectroscopy and the cumulative percentage of total isotopic dose metabolized over two hours was calculated. A previous set of healthy volunteers (N=19) who were studied in an identical fashion served as controls.*Results*: We observed decreased oxidation of 1-^{13}C-propionate to $^{13}CO_2$ in PA patients (7.6 ± 8.9% of cumulative dose oxidized, mean ± SD) compared to healthy volunteers (32.9 ± 3.2%, mean+SD, P<0.0001). Homozygosity or compound heterozygosity for nonsense variants in *PCCA* or *PCCB* was associated with lower 1-^{13}C-propionate oxidation (5.7 ± 1.7%, mean+SD) compared to patients with at least one splice-site pathogenic variant (22.6 ±11.9%, mean+SD, P=0.0071). 1-^{13}C-Propionate oxidation also correlated with biochemical parameters including plasma propionylcarnitine (R^2= 0.46, P=0.0028) and plasma total methylcitrate (R^2= 0.39, P= 0.0076). *In-vivo* 1-^{13}C-propionate oxidation correlated with height z-scores (R^2=0.47, P=0.0023) and full-scale intelligence quotients (R^2=0.55, P=0.0016). Finally, patients with hearing loss also had a reduced 1-^{13}C-propionate oxidation (7.8±4.3%, mean+SD) compared to patients with unaffected hearing (26.6±15.9%, mean+SD, P=0.0018).*Discussion*: *In-vivo* 1-^{13}C-propionate oxidation differentiated controls from patients with PA and patients with severe genotypes from those with splice variants. The cumulative oxidation of 1-^{13}C-propionate correlated with genetic, biochemical, and clinical parameters of PA, supporting its utility as a surrogate endpoint in future therapeutic trials aiming to improve clinical outcomes by resorting activity of propionyl-CoA carboxylase.

246. A Natural History Study Informs the Development of Outcome Parameters for Propionic Acidemia

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Introduction: Propionic acidemia (PA), a deficiency of propionyl-CoA carboxylase, has variable clinical manifestations. Current metabolic management is limited to carnitine supplementation and dietary restriction of protein intake. The spectrum of complications and relationships to enzymatic, molecular and clinical phenotypes remain to be fully defined. *Methods*: We have initiated a prospective study of the natural history, physiology, microbiome and biochemistry of PA at the NIH (ClinicalTrials.gov Identifier: NCT02890342). Protocol visits are accomplished via a dedicated hospital admission and include specialty evaluations, nutritional assessments, imaging studies, laboratory testing, biobanking of plasma, serum, urine, DNA, RNA, and the generation of EBV and/or fibroblast cell lines. *Results*: 20 PA patients (ages 3 - 52 years, 13 females (65%) have been evaluated. Diagnosis was confirmed molecularly in 18 patients (8 with pathogenic variants in *PCCA* and 10 in *PCCB*). Six (30%) had a height z-score <-2, 9/20 patients (45%) were overweight or obese. The whole-body bone mineral density z-score adjusted for height was <-2 in 7/15 patients (47%) (mean ± SD, -1.8 ± 1.1). In 11/20 patients (55%), total protein intake exceeded 120% of the age-adjusted recommended dietary allowance (range 46-205%, mean 121%). Fifteen of 20 patients (75%) received medical foods and 10/20 (50%) had a G-tube. Six of 20 patients (30%) received propionogenic amino acid supplements. On ECG, 9/20 (45%) had non-specific repolarization abnormalities. Seven of 20 (35%) had a QTcB interval greater than the sex- and age-specific cutoffs. Four of 19 patients (21%) had ejection fraction less than 52% (range 27-65%). Cystatin C-based glomerular filtration rate (eGFR) ranged between 25 and 108 mL/min/1.73 m^2 (mean ± SD, 73 ± 25). Five of 19 (26%) had chronic kidney disease (eGFR <60). Hearing loss of varying severity was present in 10/18 (55%) of patients. Subtle optic nerve pallor was present in 4/16 patients (25%). Seizures in the past 2 years or current use of antiepileptic drugs was reported in 6/19 patients (31%). Brain MRI revealed cerebral atrophy and small, nonspecific white matter lesions in 3/8 patients. Neurocognitive studies documented full-scale

intelligence quotients: normal range in 4/17 (24%) patients, mild intellectual disability in 8/17 (47%), and moderate intellectual disability in 3/17 (18%). 7/ 17 (41 %) were diagnosed with autism spectrum disorder. 17 patients underwent an *in vivo* assessment to measure 1-^{13}C-propionate oxidization, a whole-body measurement reflective of residual propionyl-CoA carboxylase activity. Among the many associations noted between the clinical parameters and laboratory measurements, 1-^{13}C-propionate oxidization correlated most strongly with the full-scale intelligence quotient ($r = 0.75$, P value = 0.0008), and the age-adjusted height z-score ($r = 0.69$, P value = 0.002). *Conclusions*: In summary, our experience further elucidates the natural history of propionic acidemia and measures the frequency of long-term complications, including cardiomyopathy, chronic kidney disease, hearing loss, and intellectual disability. The clinical and laboratory data collected in this study will help inform the design of future clinical trials, and especially genetic therapies, for propionic academia.

247. Correction of the "Asian Flush Syndrome" (Aldehyde Dehydrogenase 2 Deficiency) by AAV-Mediated Gene Therapy

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The "Asian Flush syndrome," presenting as alcohol-induced facial flushing, tachycardia, nausea, and headaches, is caused by aldehyde dehydrogenase 2 (ALDH2) deficiency, the most common hereditary enzyme deficiency, affecting 8% of the world population and 35-40% of the East Asian population. The most common genetic variant, known as the ALDH2*2 allele, is caused by a glutamic acid-to-lysine substitution at position 487 (E487K). ALDH2 is a key enzyme in ethanol metabolism; the ALDH2*2 mutation reduces the oxidizing ability of the enzyme, resulting in an accumulation of toxic acetaldehyde that causes the characteristic symptoms. Importantly, ALDH2*2 heterozygotes have a 7-12-fold increased risk for development of upper digestive tract cancer (oral cavity, esophagus, larynx, pharynx) that is compounded by smoking and drinking alcohol. We hypothesized that a one-time administration of an adeno-associated virus (AAV) serotype rh.10 gene transfer vector expressing the coding sequence of the human ALDH2 gene (AAVrh.10-hALDH2-HA, HA tagged to facilitate analysis) would correct the ALDH2 deficiency state on a persistent basis. To assess our hypothesis, we intravenously administered AAVrh.10-hALDH2-HA vector at 10^{11} genome copies (gc) into 6-10 wk old ALDH2 knockout homozygous (-/-) mice (n=10/group). Because alcohol consumption in the East Asian population is dominated by males, we utilized male ALDH2-/- mice for all studies. Liver hALDH2 mRNA was assessed by TaqMan and protein expression by Western analysis and immunohistochemistry. ALDH2 knockout (-/-) mice had undetectable liver levels of ALDH2 and enzymatic activity that was reversed by AAVrh.10-hALDH2-HA therapy. Four wk after therapy, the acute reaction to ethanol given by oral gavage (4 g/kg body weight) was evaluated using a panel of behavioral tests, including behavior score, open field chamber test, sectioned beam walk, skinny beam walk, skinny rod walk and screen climb. Treated ALDH2 knockout (-/-) mice performed better in all of the behavioral tests and showed fewer symptoms of acetaldehyde toxicity in the 24 hr after ethanol exposure than mice administered control vector (behavior score at 6 hr time point: p=0.01 knockout (-/-) treated *vs* knockout (-/-) control, p=0.21 knockout (-/-) treated *vs* wild type control). Taken together, these results suggest that *in vivo* AAV-mediated ALDH2 therapy may be able to reverse the ALDH2 deficiency in ALDH2*2 individuals.

248. Abstract Withdrawn

Musculo-Skeletal Diseases I

249. LNA and 2'-MOE Gapmer-Mediated Knockdown of *DUX4* in *In Vitro* and *In Vivo* Models of Facioscapulohumeral Muscular Dystrophy

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Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant disorder that manifests as a progressive wasting of muscles in the face, shoulders, and eventually, the lower extremities. It is prevalent in 1:8000 to 1:22000 people worldwide. There is no available treatment for FSHD. FSHD is caused by aberrant expression in muscle of *DUX4*, a gene normally expressed in early development but is silenced in the course of differentiation. As *DUX4* is thought to be the main pathogenic cause of FSHD, emerging therapies are typically concerned with inhibiting its expression. This study aims to develop such FSHD therapeutics to knockdown *DUX4* using antisense oligonucleotides with the locked nucleic acid (LNA) or 2'-*O*-methoxyethyl (2'-MOE) gapmer chemistry. Gapmers specifically hybridize to target mRNAs and then degrade them via an RNase H1-mediated mechanism, thereby reducing target gene expression. Immortalized FSHD patient-derived muscle fibers were lipotransfected with 100 nM of these gapmers, with the expression levels of *DUX4* and its downstream targets subsequently assessed the following day. Six out of seven designed LNA gapmers and all three designed 2'-MOE gapmers significantly reduced *DUX4* expression, with up to nearly 100% knockdown observed. Corresponding significant decreases in the expression of *ZSCAN4*, *TRIM43*, and *MBD3L2*, which are typically activated by *DUX4*, were found as well. Two successful LNA gapmers, LNAs 1 and 4, were further tested *in vivo* using *FLExDUX4* mice, an FSHD mouse model that has leaky expression of human *DUX4* even without transgene induction. Three intramuscular injections of each of these LNAs, administered every other day, into the tibialis anterior of *FLExDUX4* mice significantly knocked down *DUX4* expression compared to saline-treated controls. In summary, our designed gapmers are capable of significantly reducing *DUX4* expression in patient-derived cells *in vitro*, as well as *in vivo* in the case of the LNA gapmers. These indicate

the promising potential our gapmers have for FSHD treatment. Further assessment of therapeutic efficacy and safety using an intravenous route of administration *in vivo* will be conducted. In the long run, our study should help identify a drug candidate for FSHD therapy that could potentially enter human clinical trials.

250. Demonstration of SGCA Expression and Related Outcomes in Phase I/IIa Safety Isolated Limb Perfusion Trial in LGMD2D Subjects

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Introduction: A previous phase I, intramuscular proof-of-principle clinical trial, demonstrated robust alpha-sarcoglycan gene expression in limb-girdle muscular dystrophy, type 2D (LGMD2D), mediated by AAV under control of a muscle specific promoter (tMCK) (Neurology 2010). Following this trial, strategies to potentially achieve clinically meaningful outcomes were considered. Delivery of rAAV carrying the *SGCA* gene via an intravascular route seemed most likely to prolong ambulation. We chose to deliver self-complementary (sc) AAVrh74.tMCK.hSGCA via an isolated limb perfusion (ILP) protocol adopted from treatment of soft tissue sarcomas (Verhoef C, et al 2007). This approach was particularly well suited for gene delivery for LGMD2D because clinical weakness is predominant in the lower extremities and gene replacement to the heart is of little concern because of absent cardiomyopathy. Clinical trial feasibility was also enabled by the reduced demand for viral loading with perfusion and gene replacement exclusive to the lower limbs. Additionally by using a dwell time following ILP delivery transduction and expression of the mutant protein could be enhanced, potentially reversing the clinical disability. Pre-clinical proof of principle ILP gene transfer to non-human primates and αSG-deficient mice demonstrated efficacy. **Methods:** Five LGMD2D ambulatory subjects, ages 9-15 were enrolled in an open-label dose escalation clinical trial. The FDA mandated that ILP (sc) AAVrh74.tMCK.hSGCA gene delivery ($1X10^{12}$ vg/kg) be performed on one-limb in a non-ambulatory adult subject to establish safety before treating children. Ambulatory subjects received bilateral ILP gene delivery with either 1×10^{12} vg/kg per limb (n=3) and 3×10^{12} vg/kg per limb (n=2). The primary outcome for the clinical study was safety. The secondary outcome was improvement in the 6-minute-walk-test (6MWT) with quantitative measure of muscle strength (MVICT) in quadriceps (knee extensors; KE) as confirmatory exploratory measures. **Results:** Subjects receiving low dose ILP ($1X10^{12}$ vg/kg per limb) have completed 2 years of follow up. One low dose subject lost ambulation from a fall. Subject 3 (low dose), and subjects 4 and 5 (high dose) were stable on the 6MWT without an increase in muscle strength by MVICT. Patient 6, the youngest in the trial showed the most improvement on the 6MWT. She has now completed one-year of follow up and increased 6MWT distance by 9% (571m to 623m) without change in KE strength. Quadriceps muscle biopsies in all subjects demonstrated SGCA increased expression by immunofluorescence staining and western blot analysis, accompanied by increased muscle fiber diameters. The side effect profile was limited to groin hemorrhage and discomfort post gene delivery. **Conclusions:** ILP gene therapy demonstrated expression following vascular delivery. There was a suggestion of improvement in the youngest patient and potentially this could be improved by increasing viral dose. However, given the recent success of higher titers of AAV delivered by intravenous systemic dosing and the ability to treat all muscle, ILP may have limited application.

251. Dystrophin Restoration in a Humanized Mouse Model of Duchenne Muscular Dystrophy by Gene Editing with *S. Aureus* Cas9

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Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disease characterized by loss of ambulation and premature death often due to respiratory and cardiac complications. The disease results from deleterious mutations in the dystrophin gene that disrupt the open reading frame and cause a loss of functional dystrophin protein. Becker muscular dystrophy (BMD) is similar to DMD as it also results from mutations in the dystrophin gene. However, unlike in DMD, the mutation in a BMD genotype maintain the correct reading frame and result in production of an internally truncated, but partially functional, dystrophin proteins. The BMD phenotype is often less severe than DMD, thus converting the DMD genotype to a BMD genotype by restoring the open reading frame in the dystrophin gene is a commonly explored strategy for a potential DMD therapeutic. Genome engineering tools, such as CRISPR/Cas9, are utilized to target specific loci to create precise changes in a DNA sequence. Previously, our lab has used *S. pyogenes* Cas9 to restore dystrophin protein expression in immortalized myoblasts from DMD patients by targeting gRNAs to the intronic regions around exon 51 in the dystrophin gene, which restored the correct reading frame. Further, we have also applied *S. aureus* Cas9 (SaCas9), which has a smaller size better suited for the limited AAV packaging capacity, to remove exon 23 of the mouse dystrophin gene in the *mdx* mouse model of DMD to restore dystrophin expression and improve muscle function *in vivo*. This work demonstrated proof-of-principle of use of a CRISPR-based therapeutic for DMD that is capable of ameliorating the dystrophic phenotype. However, these CRISPR/Cas9 systems target the mouse dystrophin gene in regions that are not perfectly conserved in the human dystrophin gene. Nevertheless, preclinical development of a CRISPR/Cas9 system targeted to the human dystrophin gene should be evaluated in animal models. Hence, we have continued this work by developing an SaCas9 system targeted to remove exon 51 in the human dystrophin gene to be evaluated *in vitro* and in a novel humanized mouse model *in vivo*. SaCas9 gRNAs were screened for activity levels

in vitro in HEK293T cells and immortalized human myoblasts from DMD patients. The expected deletion of exon 51 was confirmed by end point PCR, and quantified by droplet digital PCR of the genomic DNA and dystrophin cDNA. Protein lysates from treated DMD myoblasts in differentiation culture were analyzed by Western blot to confirm restoration of dystrophin protein expression. In order to test the system targeted to the human dystrophin gene, we developed a novel dystrophic mouse model that harbors the human dystrophin gene but lacks exon 52, creating a DMD genotype that is correctable by removal of human exon 51. We confirmed lack of dystrophin protein expression in these mice by immunohistochemistry staining and Western blot. After systemic treatment of adult mice by intravenous administration of AAV vectors encoding the SaCas9 system, we detect deletion of exon 51 in the genomic DNA and mRNA. Similarly, we observe moderate dystrophin protein restoration by western blot and immunohistochemistry. We are assessing the dystrophic phenotype of this mouse after systemic treatment with our CRISPR/SaCas9 system. We are also comparing levels of gene editing following treatment of neonatal or adult mice. Ongoing studies will quantify improvements to the dystrophic phenotype and determine efficacy of our CRISPR/SaCas9 system *in vivo* to restore dystrophin protein and improve muscle function.

252. Mitotalens as Genetic Tools to Reduce Mutant Mitochondrial DNA Levels in a Mouse Model of Heteroplasmic mtDNA Mutation

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Introduction: Mitochondrial DNA (mtDNA) mutations mainly cause diseases when the levels of the mutated genomes exceed a threshold that triggers a bioenergetics defect. We have designed mitochondrial-targeted, Transcription Activator-Like Effector Nucleases or mitoTALENs to cleave specific sequences in the mtDNA with the final goal of reducing the levels of mtDNA carrying pathogenic mutations. We have previously shown that mitoTALENs could successfully reduce the levels of several pathogenic mtDNA mutations in cultured human cells. We now show that the approach is also effective in a mouse model. Methods and Results: We developed a mitoTALEN specific for the mouse mitochondrial gene coding for the $tRNA^{ALA}$ mutation, C5024T. Mice carrying high levels of this pathogenic mtDNA mutation have reduced body mass and develop cardiomyopathy at older age. The mice also show decreased levels of $tRNA^{ALA}$ in heart and skeletal muscle. The mitoTALEN constructs included an N-terminal mitochondrial localization signal, an immuno-tag, and a fluorescent marker. MitoTALENs localized to mitochondria and reduced the levels of the pathogenic mtDNA in cultured MEFs from this mouse. Mitochondrial oxidative phosphorylation protein levels were recovered 2 weeks after transfection with the mitoTALENs. We have designed and produced shorter versions of the mitoTALEN for the C5024T mutation, reducing the size of the RVDs and changing the MLS to be able to comfortably pack each gene coding for a mitoTALEN monomer in a AAV2/9 vector. Intramuscular injection of the tibialis anterior (TA) with AAV9 particles coding for both monomers showed high expression of both mitoTALENs monomers and led to a significant reduction in the levels of the pathogenic C5024T mtDNA mutation, when compared to the opposite leg injected with the control AAV9-GFP. This was observed at 4, 6, 10, 12 and 24 weeks after injection. As a biochemical improvement, $tRNA^{ALA}$ levels were recovered in the TA after the mitoTALEN injections. No depletion or non-intended deletions were found after the mitoTALEN treatment. Systemic injections with the AAV2/9-mitoTALENs monomers also showed consistent decrease of the mutant mtDNA in muscle and heart. Conclusions: Our findings demonstrate that AAV9-delivered mitoTALENs promote a stable shift in mtDNA heteroplasmy in muscle after IM injection and in cardiac and skeletal muscle tissues after systemic injection, bringing the use of mitochondrial nucleases closer to clinical trials.

253. Systemic Gene Therapy Restores γ-Sarcoglycan Expression in Skeletal and Cardiac Muscle in LGMD2C Mice

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LGMD2C is caused by gamma-sarcoglycan (SGCG) deficiency. The sarcoglycans (α, β, γ, and δ-SG) are structural proteins localized at the membrane of muscle cells that together with dystrophin and other proteins make up the dystrophin-associated protein complex (DAPC). The lack of SGCG and loss of other components of the DAPC results in increased permeability and membrane tears. This leads to myofiber degeneration, chronic inflammation, and elevated creatine kinase (CK) levels. Like other sarcoglycanopathies, LGMD2C presents as a progressive muscular dystrophy affecting the hip and girdle muscles before spreading to lower and upper extremity muscles. Presentation typically occurs in childhood. Corticosteroids are used in some patients for limited symptom management and while exon skipping is being explored as a therapeutic approach, pre-clinical studies are still in progress, clinical efficacy will require targeting specific mutations, and treatment requires ongoing administration. Thus, there is no current form of LGMD2C treatment. Given the small size of *SGCG* and the potential to fully restore function, gene replacement therapy is the ideal therapeutic candidate. The goal of this study is to provide pre-clinical, proof-of-principle safety and efficacy data for scAAVrh74.MHCK7.hSGCG, codon optimized, gene transfer in SGCG knock-out (SGCG-/-) mice with delivery of $5x10^{13}$ vg/kg. The SGCG-/- mouse has clinical-pathological features that replicate the human muscle disease, making it an ideal model for translational studies. The methods used were very similar to those applied for LGMD2E. Systemic delivery through the tail vein of SGCG knock-out mice provided a rationale for delivery in a clinical trial that would lead to clinically meaningful results. Initial efficacy studies indicated tail vein injection of scAAVrh74.MHCK7.hSGCG resulted in widespread transgene expression in muscles throughout the hindlimbs, forelimbs, torso, and the heart, which was accompanied by improvements in histopathology including reduction in central nucleation and increased fiber diameter and restored other components of the dystrophin-associated protein complex. Additional outcome measures being investigated include

fibrosis quantification as indicated by Sirius red staining for collagen deposition, force production and resistance to injury of the tibialis anterior and diaphragm muscles, the degree of kyphoscoliosis of the spine, and overall activity of the mice measured by laser monitoring of open-field cage activity. Delivery of a normal copy of the *SGCG* gene to diseased muscle will allow for production of functional wild-type protein, reversal of disease pathology, and an improvement in muscle function. This pre-clinical study is pivotal for establishing proof-of-principle for translation of AAV.SGCG gene transfer in LGMD2C patients.

254. Feasibility of Lentiviral Vector Gene Therapy for the Treatment of *TCIRG1*-Mutated Autosomal Recessive Osteopetrosis

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Autosomal recessive osteopetrosis (ARO) is a group of rare genetic diseases, affecting osteoclast differentiation or function. The majority of ARO patients (55%) presents mutations in *TCIRG1* gene, encoding the a3 subunit of V-ATPase proton pump, which is necessary for bone resorption activity. Osteoclast dysfunction results in limited bone marrow cavity, causing extramedullary hematopoiesis and increased number of circulating $CD34^+$ cells in patients. Symptoms include dense and brittle bones, anemia and progressive nerve compression, leading to death in the first decade of life. To date, bone marrow transplantation is the only cure for ARO patients, but its applicability is limited by availability of HLA-matched donor and toxicity of conditioning regimens. Thus, gene therapy (GT) may represent an alternative therapeutic option for these patients. To this end, we generated two lentiviral vectors, driving *TCIRG1* expression under the control of the *PGK* promoter. The first vector has been clinically optimized, while in the second one the *dNGFR* gene was added as a reporter. We transduced Lin- cells isolated from the spleen of 10-12 day old *Tcirg1*-mutated mice, the *oc/oc* spontaneous model characterized by a severe osteopetrotic phenotype. Lin- cells were differentiated *in vitro* on dentine slices. Transduced cells showed a partial rescue of osteoclasts resorptive function with both vectors. Transduced Lin- cells were also injected intra-liver into irradiated *oc/oc* newborn mice. Differently from untreated *oc/oc* mice that have a life expectancy of 2-3 weeks, GT mice showed improved lifespan and absence of circling behavior. However, they had a smaller body weight compared to littermates. In parallel, we transduced and expanded CD34+ cells isolated from the peripheral blood of six ARO patients. Cells were differentiated *in vitro* towards the myeloid lineage and then into osteoclasts. We observed the formation of TRAP-positive multinucleated osteoclasts from both transduced and untransduced CD34+ cells, as expected. Importantly, bone resorption capacity was restored in cells transduced with our corrective lentiviral vectors. Moreover, we transplanted expanded ARO CD34+ cells into NSG mice to evaluate their repopulating capacity. We assessed that transduced and expanded ARO CD34+ cells were able to engraft long-term in NSG mice, showing up to 30% of human chimerism in peripheral blood 13 weeks after transplantation. In conclusion, our results suggest that gene therapy could represent a feasible, readily available treatment for ARO patient to whom HLA-matched donor is not available.

255. AAV9-Mediated Gene Delivery of GDF11 Propeptide-Fc Induces Skeletal Muscle Hypertrophy

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Muscle atrophy is associated with a variety of conditions including muscular dystrophy, mitochondrial myopathy, chronic kidney disease and aging-associated sarcopenia. Strategies aimed at modulating the transforming growth factor beta (TGFβ) signal transduction pathway in muscle tissue have shown promise in mitigating muscle deterioration and weakness in muscle atrophy. Growth and differentiation factor 11 (GDF11; BMP11) is a notable cytokine in the TGFβ superfamily, and there is emerging evidence to suggest GDF11-mediated activation of SMAD2/3 via activin type I receptor binding induces skeletal muscle atrophy. With this in mind, we hypothesized that inhibition of endogenous GDF11 by the GDF11 propeptide would lead to skeletal muscle hypertrophy. In this study, we aimed to characterize the effects of the GDF11 propeptide on skeletal muscle mass and function. To extend factor half life *in vivo*, the gene encoding the Fc region from human IgG1 was fused to the 3'-end of the human GDF11 propeptide gene to generate the GDF11Pro-Fc-1 construct. The full-length transgene driven by the ubiquitous CAG promoter was then packaged into adeno-associated virus serotype 2/9 vector (AAV9-GDF11Pro-Fc-1). We evaluated the effects of AAV9-GDF11Pro-Fc-1 after both systemic and local treatment. For systemic administration, 3 day old neonatal C57BL/6J mice (n=5) were administered a single dose of $5x10^{11}$ vg/mouse AAV9-GDF11Pro-Fc-1 or PBS by intravenous (IV) injection via the temporal vein. To assess local effects, 8-9 week old C57BL/6J mice (n=5) were treated with a single dose of $1x10^{12}$ vg/kg AAV9-GDF11Pro-Fc-1 or a control vector delivered by intramuscular (IM) injection into the tibialis anterior (TA) and gastrocnemius (Gas) on the right side. Total body mass, forelimb/hindlimb grip force and treadmill running endurance were assessed at regular intervals. IV-injected and IM-injected mice were sacrificed at 28 weeks and 10 weeks post-treatment, respectively. A sustained increase in total body mass was observed starting 2 weeks post-treatment in mice administered AAV9-GDF11Pro-Fc-1 systemically. Additionally, an increase in forelimb/hindlimb grip force, limb muscle mass and myofiber minimum feret diameter was seen after both systemic and local treatment with AAV9-GDF11Pro-Fc-1. However, no difference was detected in treadmill running endurance. Heart mass was also unaltered by treatment with AAV9-GDF11Pro-Fc-1. Correct protein expression of GDF11Pro-Fc-1 *in vivo* was confirmed by Western blot. From these results, we determine that AAV9-GDF11Pro-Fc-1 induces skeletal muscle hypertrophy without causing cardiac hypertrophy.

These results support the further study of the GDF11 propeptide for therapeutic applications in muscle atrophy, and a trial in dystrophic mdx mice is currently in progress.

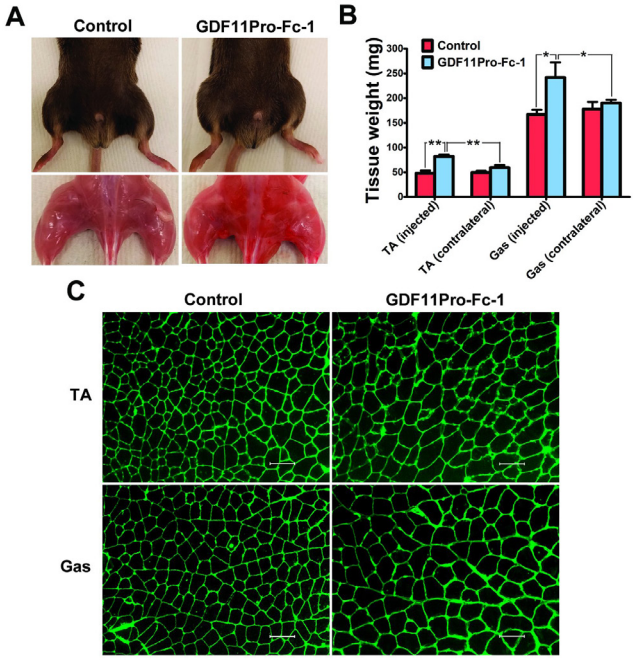

Figure 1. Local delivery of AAV9-GDF11Pro-Fc-1 by IM injection results in muscle hypertrophy at the injection site. (A) Gross hindlimb musculature. (B) Tissue mass of TA and Gas muscles (n=5; *=p<0.05, **=p<0.01). (C) Immunofluorescence images of TA and Gas muscle sections stained with Alexa Fluor 488-conjugated wheat germ agglutinin to visualize muscle fibers (scale bar = 100 μm).

256. Abstract Withdrawn

257. CRISPR/Cas9-Mediated Gene-Editing Rescues Dystrophin Expression in a Dog Model of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a lethal and devastating genetic disorder caused by a lack of dystrophin protein due to mutations in the *DMD* gene. DMD is mostly caused by out-of-frame mutations, leading to a premature stop codon. Most gene-editing studies currently aim to remove a part of the *DMD* gene and produce a truncated but functional dystrophin protein to shift the clinical prognosis towards its milder counterpart, Becker muscular dystrophy (BMD). Previous studies showed that gene-editing restored dystrophin expression and improved the phenotype of DMD model mice. Although DMD mouse models (e.g. mdx) are most commonly used, they have several limitations. First, most DMD mouse models show a very mild phenotype, especially in the heart. In fact, many therapeutic approaches effective in mdx mice have failed to translate in large animals because of this issue. Second, due to their small size, the clinical and safety assessments cannot be conducted as detailed as in large animal models. Third, mdx mice harbor a nonsense mutation in exon 23, which is extremely rare in human DMD patients. In contrast, dystrophic dogs harbor a mutation in intron 6, which is within the N-terminal mutation hotspot. In fact, approximately 7% of DMD patients have mutations within exons 3-9 in this region. Here, we sought to examine the effects of gene-editing in a DMD dog model, the Canine X-linked Muscular Dystrophy (CXMD). To restore the reading frame of the CXMD, at least 3 exons, exons 6-8, need to be removed by non-homologous end joining (NHEJ). We employed CRISPR/*Staphylococcus aureus* Cas9 (SaCas9) system and designed five guide RNAs targeting intron 5 and six guide RNAs targeting intron 8 using the PAM sequence 5'-NNGRRT-3'. To select the most effective pair of guide RNAs for the removal of the targeted region in the genome, each guide RNA was introduced into MDCK cells (dog kidney cells) for T7E1 assays. The T7E1 assay showed that three guide RNAs targeting intron 5 and two guide RNAs targeting intron 8 efficiently induced site-specific DNA double-strand breaks in genomic DNA. Next, we examined the effects of two SaCas9 vectors (one targeting intron 5, the other targeting intron 8) transfected into primary CXMD dog muscle cells. We demonstrated that the gene-editing using these guide RNAs and SaCas9 deleted the targeted region in the genome and rescued in-frame dystrophin expression (exon 6-9 skipped) efficiently in the DMD model dog cells in vitro. Currently, we are testing the in vivo efficacy of SaCas9-mediated gene editing after intramuscular AAV9 delivery to tibialis anterior muscles of the dog model.

258. Exosomes Generated from Umbilical Cord-Derived Stem Cells Reduce Intervertebral Disc Degeneration

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BACKGROUND: Cell-based therapies for intervertebral disc degeneration (IVD) have been widely studied. Cell-Cell communication is based on a paracrine, connexons or cell-cell contact. It has been recently demonstrated that mesenchymal stem cells derived from multiple tissue types release extracellular vesicles termed exosomes that provide therapeutic benefits. The purpose of this study was to explore the role of exosomes derived from umbilical cord-derived mesenchymal stem cells (UC-MSCs) their protective effect in a rabbit IVD model and therapeutic effects of miRNAs. **METHODS:** Exosomes secreted by UC-MSCs were purified by differential centrifugation and identified by transmission electron microscope and analysis of exosomal specific marker proteins after hypoxic cell culture. Fluorescence microscopy was used to examine the uptake of exosomes by recipient cells. Western blot analysis was used to examine proteins such as aggrecan, sox-9, collagen II and hif-1α in the UC-MSCs. The therapeutic benefits of UC-MSC exosomes were studied by Intradiscal injection. Analysis of IVD degeneration was studied 8 weeks post-transplant in a rabbit IVD degeneration model. **RESULTS:** UC-MSCs secreted exosomes and are able to be injected intradiscally into a degenerated rabbit disc model. 8 weeks after transplantation the. Percent disc height index (%DHI) was measured every week and histology of injured disc was evaluated at 8

weeks after transplantation. IVD degeneration was significantly lower in the UC-MSC exosome group as compared to the no-injection group. Analysis revealed that the %DHI in the UC-MSCs secreted exosomes group was significantly higher than that in the control group at 8 weeks after transplantation 79% vs. 55% ($p \leq 0.05$). Histological analysis revealed the formation of nucleus pulposus (NP)-like tissue at the outer layer of annulus fibrosus was frequently observed in the UC-MSCs group but not in the control group, indicative of extracellular matrix remodeling. **CONCLUSION:** Our study indicates that exosomes are an important vehicle in information exchange between UC-MSCs and their intended target tissue. The potential use of UC-MSC exosomes as a cell-free product offers several potential advantages that can provide regenerative effects. In Summary, we have for the first time demonstrated the therapeutic benefits of UC-MSC exosomes in. a rabbit IVD degeneration model.

259. Correction of a Common Pseudoexon-Creating Mutation in the *COL6A1* Gene Utilizing Both Single and Dual Guide RNA-Mediated CRISPR/Cas9 Approaches

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Collagen VI-related dystrophies (COL6-RD) are a group of congenital neuromuscular disorders of muscle and connective tissue, for which there are no effective treatments. COL6-RDs are caused by mutations in any of the three COL6 genes; *COL6A1*, *COL6A2* and *COL6A3* encode the collagen α1, α2 and α3 (VI) chains respectively, which undergo hierarchical assembly to produce the extracellular matrix protein collagen type VI. Collagen VI is primarily produced by muscle interstitial fibroblasts (MIF). Recently, we discovered a novel but common *de novo*, heterozygous mutation (c.930+189C>T) in intron 11 of the *COL6A1* gene. This mutation creates a splice donor site and prompts insertion of an in-frame pseudoexon in mature mRNA, that is translated to exert a severe dominant negative on collagen VI matrix assembly. As proof-of-principle, we previously showed that skipping of the pseudoexon with antisense oligonucleotides dramatically improved collagen VI matrix deposition. Given its location in the deep-intronic region of intron 11, the +189C>T mutation is a unique candidate for gene editing approaches that could eliminate the deep-intronic mutant splice site and restore normal splicing. Here we devised two gene-editing strategies to target the +189C>T mutation: one involving mutation-specific gRNAs to generate random edits in regions proximal to the mutation site, and a second utilizing paired gRNAs to induce an intronic deletion encompassing the mutation site. For both strategies, gRNAs were designed and cloned into an spCas9-GFP plasmid. Following transfection of gRNA/Cas9-GFP in patient-derived primary dermal fibroblasts, we sorted GFP+ cells and plated them using a serial dilution method to isolate and grow clonal populations. Sanger sequencing, RT-PCR and qPCR were used to determine pseudoexon mRNA expression and identify clones that underwent favorable edits. Matrix secretion and deposition was assessed by immunofluorescence staining for collagen VI microfibrils. Utilizing the single gRNA approach, we isolated a clonal cell population with favorable edits that significantly reduced the expression of the pseudoexon. This was accompanied by an improvement in the quality of the collagen microfibrillar matrix *in vitro*. A drawback of the single guide RNA strategy was that the cut sites were slightly further away from the mutation and long edits overlapping with the mutation site were not frequently observed. The dual guide RNA approach is therefore more promising for clinical translation as it seeks to eliminate the mutation bearing genomic fragment at a higher frequency. Previous studies by our group and others show that patients fully heterozygous for dominant *COL6* mutations have a more severe clinical course of disease compared to those who exhibited somatic mosaicism. While single and dual CRISPR/Cas9 strategies in their current stage of development will likely not generate favorable edits in every MIF, we believe that reducing the load of mutant collagen chains produced by the MIF population *in vivo* can achieve a state of somatic mosaicism and provide symptomatic relief. Along with MIF-targeting viral vector delivery approaches we next seek to develop *in vivo* CRISPR/Cas9-mediated elimination of the common deep-intronic +189C>T mutation.

260. AAV1.NT-3 Increases Muscle Fiber Diameter through Activation of mTOR Pathway and Metabolic Remodeling in a CMT Mouse Model

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AAV1.NT-3 enhances muscle fiber diameter through activation of mTOR pathway and metabolic remodeling in a CMT mouse model Neurotrophin 3 (NT-3) has well-recognized effects on peripheral nerve and Schwann cells, promoting axonal regeneration and associated myelination. In previous studies, we showed that AAV1-NT-3 gene therapy improved motor function, histopathology, and electrophysiology of peripheral nerves in TremblerJ (Tr^J) mouse model of CMT1A (Charcot-Marie-Tooth Disease/ type1A). In this study, we assessed the effects of AAV.NT-3 gene therapy on the oxidative state of the neurogenic muscle from the Tr^J mice at 16 weeks post-gene injection and found that the muscle fiber size increase was associated with a change in the oxidative state of muscle fibers towards normalization of the fiber type ratio seen in the wild type. NT-3-induced fiber size increase was most prominent for the fast twitch glycolytic fiber population. These changes in the Tr^J muscle were accompanied by increased phosphorylation levels of 4E-BP1 and S6 proteins as evidence of mTORC1 activation. In parallel, the expression levels of the mitochondrial biogenesis regulator PGC1α, and the markers of glycolysis (HK1 and PK1) increased in the Tr^J muscle. *In vitro* studies showed that recombinant NT-3 can directly induce Akt/mTOR pathway activation in the TrkC expressing myotubes but not in myoblasts. In addition, myogenin expression levels were increased in myotubes while $p75^{NTR}$ expression was downregulated compared to myoblasts, indicating that NT-3 induced myoblast differentiation is associated with mTORC1 activation. These studies for the first time have shown that NT-3 increases muscle fiber diameter in the neurogenic muscle through direct activation of mTOR pathway and that the fiber size increase is more prominent for fast twitch glycolytic fibers.

261. Systemic rAAV9-Microdystrophin Transduction with MSCs Pre-Treatment Improve Transgene Expression and DMD Phenotype in Canine X-Linked Muscular Dystrophy

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Background: Duchenne muscular dystrophy (DMD) is a congenital disease causing progressive deterioration of skeletal and cardiac muscles because of mutations in the dystrophin gene. Supplementation of dystrophin using rAAV-microdystrophin is sufficient to improve pathogenesis of animal models of DMD. However, we previously reported that local injection of rAAV2 or rAAV8 into canine skeletal muscles without immunosuppression resulted in insufficient transgene expression with potent immune responses. Here we used mesenchymal stromal cells (MSCs) to investigate strategies of inducing immune tolerance to the rAAV9 vector and transgene expression. MSCs have been employed in various inflammatory diseases including graft-versus-host disease (GvHD) by their immunosuppressive effects. Furthermore, immune modulating effects of MSCs on rAAV transduction were examined. **Methods:** Bone marrow-derived MSCs and rAAV9-luciferase or rAAV9-microdystrophin were intravenously injected into the normal dog or $CXMD_J$ at eight weeks old. Seven days after injection, MSCs were systemically injected again. At eight days after the first injection, rAAV9-luciferase or rAAV9-microdystrophin were intravenously injected into the same dog. To examine the immune response against rAAV9, IFN-γ expression in the purified canine peripheral leukocytes stimulated with rAAV was analyzed using qRT-PCR. Expressions of the transgene in skeletal muscles of the rAAV9-luciferase or rAAV9-microdystrophin transduced animals were confirmed by immunohistochemistry. Evasions of neutralizing antibodies induction against rAAV were determined at various time points of rAAV-transduced dogs. In addition, MSCs-treated $CXMD_J$ with rAAV9-microdystrophin transduction and non-treated $CXMD_J$ were compared to assess gait function and lameness of the limb. **Results:** Administration of rAAV9 following MSCs treatment resulted in higher expression of the transgene (luciferase or microdystrophin) at the skeletal muscle, compared to the rAAV transduction alone. Expression of IFN-γ in the purified peripheral blood leukocytes after the rAAV9 exposures were not enhanced in the rAAV9 with MSCs, suggesting the immune suppressive effects of the MSCs. The $CXMD_J$ treated with MSCs and rAAV9-microdystrophin showed functional improvement than other DMD dogs of the same age. **Conclusion:** Our results demonstrate that rAAV injection with MSCs pre-treatment improved expression of the rAAV-derived transgene in dogs. This strategy would be a practical approach to analyze the expression and function of the transgene *in vivo*. These findings also support the future feasibilities of rAAV-mediated protein supplementation strategies to treat DMD.

262. Lentiviral Mediated Collagen VII Expression in Allogeneic MSCS Restores Anchoring Fibrils in a Recessive Dystrophic Epidermolysis Bullosa Skin Graft Model

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Recessive dystrophic epidermolysis bullosa (RDEB) is a debilitating genodermatosis caused by loss-of-function mutations in COL7A1, the gene encoding type VII collagen (C7), a protein central for anchoring fibrils (AFs) formation at the dermal-epidermal junction (DEJ). Presently there are no curative treatments for RDEB but retroviral transduced autologous epidermal grafts are being investigated and early safety studies of intradermal lentiviral engineered fibroblast injections are underway. An alternative approach aims to modify allogenic mesenchymal stem cells (MSCs) to ultimately provide a more generalized treatment for RDEB. In this study MSCs were engineered to overexpress C7 using a third generation SIN-LV vector encoding a codon optimized COL7A1 transgene under the control of the human PGK promoter. Transduction efficiency was on average 29% (range 17-43%) by flow cytometry for C7) and proviral copy number was on average 1.5 copies per cell by qPCR. Transduction did not alter characteristic markers of MSCs with >97% of cells remaining positive for CD105, CD90 and CD73 markers and retaining their differentiation potential. Overexpression of C7 was confirmed by FACs, IF and Western blot in both cell lysates and supernatant, indicating that genetically engineered MSCs are able to produce and secrete full-length C7. Engineered MSCs were then included in the dermal compartment of ex-vivo RDEB skin graft productions (n=5) which were then used in human:murine modelling experiments to assess basement membrane architecture. Histological assessment after 8 weeks revealed recovery of DEJ function with no signs of blister formation contrary to the grafts generated without C7-MSCs where severe blistering was observed (n=5). Species-specific antibodies and FISH analysis were used to confirm the human origin of the graft and the long-term engraftment of MSCs, respectively. This was further supported by robust expression of human-specific C7 in grafts incorporating engineered MSCs, indicating deposition of C7 throughout the DEJ. The level of C7 was significantly higher in the grafts incorporating engineered MSCs compared to RDEB grafts as measured by the corrected total area fluorescence (CTAF $6.51 \pm 4.99 \times 10^6$ SD versus $1.31 \pm 0.265 \times 10^6$ SD, $p<0.05$, n=5 for each group). Importantly, the secretion of C7 by genetically engineered MSCs translated to the formation of robust and abundant mature AFs at the levels seen in wild-type grafts, providing a surrogate for functional correction. Quantitative ultrastructural techniques confirmed that

the density of AFs in the engineered MSC grafts achieved levels comparable to WT grafts with an average of 30 +/-3 SD AFs per 10 micron, whereas in the RDEB grafts exhibited an average of 8 +/- 1.5 SD AFs per 10 micron ($p<0.000001$). The results of this study provide proof-of-concept data that lentiviral engineered MSCs could form the basis of a RDEB cell therapy and further experiments are underway to further investigate strategies for systemic delivery of C7-MSCs.

263. Dental Pulp-Derived Cell Therapy for Duchenne Muscular Dystrophy is Safe and Facilitates Physical Capabilities

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[**Background**] Duchenne muscular dystrophy (DMD) is an incurable genetic disease with early mortality that exhibits skeletal muscle weakness with chronic inflammation. Dental pulp stem cells (DPSCs) would be potential therapeutics because of their immunosuppressive properties and multipotency. Last year, we presented a strategy for systemic transplantation of DPSCs into DMD dog. In the present study, we examined the strategies for further safe and effective cell transplantation to develop a novel approach for functional recovery of the skeletal muscles using a dog model of DMD. We also investigated the effects of IL-10 transduced DPSCs on DMD treatment to enhance the immunosuppressive function. [**Methods**] DPSCs were transduced with or without IL-10/rAAV-1 and then those cells were intravenously injected into canine X-linked muscular dystrophy in Japan ($CXMD_J$) for 8-10 times. DPSCs sampled from treated dog's blood were quantified by *Alu*-PCR and the cytokine levels were measured by ELISA. Clinical phenotypes in the transplanted dogs were analyzed by using blood exams, physical capacity and MRI analysis compared with non-injected littermates as controls. [**Results**] We confirmed that cells survived in blood for up to 48 h after transplantation and also the slightly increased IL-10 were detected in parallel. No obvious abnormality related to hepato-renal damages, or anemia due to systemic administration in all dogs was observed. Compared to the untreated dogs, IL-10/DPSCs-treated $CXMD_J$ was growing better during development. Serum levels of creatin kinase and IL-6 were regulated in the DPSCs-treated $CXMD_J$. The down-regulation of inflammation in the lower legs of DPSCs-treated $CXMD_J$ was also suggested by MRI analysis. Although $CXMD_J$ showed that progressive muscle atrophy in the all four limbs, exercise intolerance and abnormal locomotion improved in the IL-10/DPSCs-treated $CXMD_J$ along with faster pace of getting up and running. [**Conclusion**] We suggested that the systemic injection of DPSCs was safe and ameliorated the progressive phenotype in $CXMD_J$. The therapeutic effects might be associated with the production of paracrine or endocrine factors, such as IL-10, that regulate inflammation and might also stimulate the proliferation of endogenous stem cells at the injured muscle tissue. This strategy of DPSCs treatment would be promising for the future DMD cell therapy.

264. Combinatorial PRG4 and IL-1Ra Gene Therapy Preserves Articular Cartilage and Prevents Pain Hypersensitivity in Osteoarthritis

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Introduction: Osteoarthritis (OA) is a complex disorder with a complicated set of underlying causes affecting millions of people. To date no disease-modifying therapies have been found, which is likely due to the multifactorial nature of disease and limitations of single therapy approaches. Among the alterations found in OA, inflammation and proteoglycan loss are known to be major findings in disease progression. Previously, we showed that overexpression of the gene PRG4 which codes for the proteoglycan Lubricin is able to significantly delay progression. We and others have also shown that IL-1Ra overexpression is capable of delaying progression by inhibiting the IL-1 inflammatory pathway. Trials with protein therapies have shown limited efficacy due to rapid turnover; therefore, we opted to test a combinatorial, helper-dependent adenoviral (HDAd) gene therapy strategy to achieve long-term expression of the two target genes in joint tissues. **Results:** We found that when compared to each individual monotherapy, combinatorial therapy with IL-1Ra and PRG4 preserved articular cartilage volume and prevented erosion as evidenced by measures of surface area of the bone covered by cartilage in both the destabilization of medial meniscus (DMM) and cruciate ligament transection (CLT) models. Furthermore, combinatorial therapy was successfully able to prevent the development of thermal hyperalgesia to at least 3.5-months after DMM surgery (later time points not yet tested) while monotherapy groups developed hypersensitivity between 2.5- and 3.5-months after surgery. Synovitis scores were low to none for all experimental groups and not significantly different, and subchondral sclerosis was observed in all surgical groups at both 2.5- and 3.5-month endpoints. **Methods:** OA was surgically induced at 8 weeks of age in male FVB mice via either DMM or CLT. HDAd containing either IL-1Ra driven by an inflammation inducible, NFkB promoter and/or PRG4 driven by a constitutive EF1a promoter was injected at 10^8 viral particles per joint two weeks after surgery. Mice underwent behavior tests including rotarod, open field analysis, grid foot slip, and hot plate nociception one week before terminal sacrifice at either 2.5- or 3.5-months after surgery. The left knee from each mouse was collected for histological scoring and immunohistochemistry, while the right knee was used for phase contrast microCT analysis to quantify articular cartilage volume, surface area of the subchondral bone covered by articular cartilage, and subchondral bone parameters. **Conclusion:** Combinatorial therapy is able to improve preservation of articular cartilage and prevent the clinically relevant development of hyperalgesia. Hyperalgesia was not correlated to changes in subchondral bone, cartilage integrity, or synovitis in this model. The low-dosage requirements combined with the other attractive features of HDAd (low immunogenicity, long-term expression, large

packaging capacity, non-integrating episomal transduction, ease of production scalability) make this a potential clinical approach for future development.

265. A Dose-Response Assessment of FKRP Gene Replacement Therapy in a Dystrophic Mouse Model

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Mutations in the fukutin-related protein (FKRP) gene cause a wide spectrum of phenotypic severities commonly referred to as muscular dystrophy-dystroglycanopathies. These loss-of-function mutations disrupt the glycosylation of α-dystroglycan (α-DG), ultimately compromising muscle membrane stability during the muscle contraction cycle. It has been previously determined that a single dose of recombinant adeno-associated virus serotype 9 (AAV9) vector expressing human FKRP administered at varying time points in disease progression is effective at improving muscle pathology and function. However, the optimal dose to sustain long-term efficacy has yet to be determined. We addressed this issue by conducting a dose-escalation study in which one of three increasing doses of AAV9-FKRP was administered to a p.Pro448Leu ($FKRP^{P448L}$) dystrophic mouse model in the early stages of disease progression. Over the 52-week study period, data were collected to assess treatment effects on respiratory function, improvement and preservation of muscle function, protein expression, and histopathology. Based on the results, the rescue of functional α-DG and thus, improvement in muscle function appears to occur system-wide, in a dose-dependent manner. In addition to preventing disease progression, treatment with AAV9-FKRP was able to extend the lifespan of dystrophic mice per additional longevity studies. These studies support the initiation of early-stage trials for FKRP-related disorders.

266. CRISPR/*Cas9*-Mediated Correction of Dystrophin Mutation Improves Muscle Progenitor Cell Bioenergetics and Stress Resistance

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Duchenne Muscular Dystrophy (DMD) is a lethal genetic disease characterized by a lack of dystrophin expression, which leads to progressive weakening and wasting of skeletal and cardiac muscles. Lack of myofiber dystrophin is generally accepted as a cause of DMD histopathology; however, DMD is also a muscle stem cell disease. Indeed, a recent study (Dumont *et al.*, 2015) demonstrated the expression of dystrophin in satellite cells and revealed its novel role as a key regulator of asymmetric cell division and function. In addition, multiple lines of evidence underline role for muscle progenitor cell (MPC) depletion/dysfunction in DMD progression. Recently, the clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein-9 nuclease (Cas9) system has emerged as a potential therapeutic tool for DMD, and several studies have shown restored dystrophin expression and, consequently, improved muscle function in skeletal muscle of *mdx* mice (a mouse model of DMD). Interestingly, most reports on gene editing describe the exclusive use of young animals. We hypothesize that the beneficial effect of gene editing will be greatly improved when combined with a strategy to replenish exhausted stem cells. We believe that therapeutics to restore dystrophin in myofibers, as well as approaches to delay MPC depletion, are both necessary to alleviate muscle weakness, especially in older DMD patients who exhibit more severe stem cell exhaustion. In the current study, we have restored dystrophin expression by editing out the mutated exon in MPCs derived from *mdx* muscle. We show that *ex vivo* dystrophin-restored MPCs (drMPCs) exhibit superior muscle regenerative ability compared to *mdx* MPCs, due to the fact that drMPCs have improved proliferative and differentiation properties and demonstrate enhanced energy production and resistance to stress. Particularly, drMPCs showed improved cellular growth under stress. Cell viability assays *in vitro* showed no changes under normal growth conditions; however, under hypoxia (1% O_2), the cellular growth of drMPCs was improved by ~40% (p=0.0004). Cell cycle analysis revealed an increase in drMPC proliferation rate. Next, we showed that dystrophin restoration led to improved mitochondrial function and repressed oxidative stress in MPCs. We evaluated the oxygen consumption rate, a measure of overall mitochondrial respiration, in drMPCs, and observed a significant increase in basal mitochondrial respiration, ATP production, and both spare and maximal respiratory capacities. In addition, the mitochondrial membrane potential ($\Delta\Psi m$) and ROS/RNS levels, which are elevated in *mdx* cells, showed notable normalization in drMPCs (40% and 10% lower, respectively). Finally, we evaluated the regenerative potential of drMPCs *in vivo,* and demonstrated improved outcomes upon transplantation of gene-corrected dystrophic MPCs and expression of dystrophin in dystrophic *mdx* skeletal muscle. Taken together, our results indicate that dystrophin restoration appears to boost MPC survival upon transplantation into the *mdx* mouse model through modulations in cellular energetics and stress resistance, indicating potential avenues to improve the outcomes of stem cell-based therapies for DMD.

267. Examination of AAV.ANO5 Treatment and Gender Effects on Anoctamin5 Mutant Mice

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Limb Girdle Muscular dystrophies (LGMDs) are a group of degenerative muscle disorders that that initially present with shoulder and hip muscle weakness but eventually affect all muscles. Of the 17 defined recessive LGMDs, LGMD type 2L is caused by mutations in

the anoctamin 5 (*ANO5)* gene. LGMD2L represents 4-20% of total recessive LGMD prevalence, and there is no treatment or cure for LGMD2L. Case studies suggest that anoctaminopathy tends to present with myalgia and mild weakness, and may be more prevalent and/or more severe in males, as compared to females. We have previously characterized an *Ano5* mutant mouse that recapitulates many aspects of LGMD2L. Given potential gender bias in the human disease, we investigated phenotypes of male and female *Ano5* mutant mice. Among the differences we found, females tended to exhibit greater exercise endurance than males, and greater activity of the mitochondrial enzyme citrate synthase. In addition, male and female mice responded distinctly to long-term antioxidant therapy. The membrane repair deficit of *Ano5* mutant mice did not show any gender bias, nor was regenerating fiber size following acute injury significantly different. Previous work also demonstrated intramuscular delivery of an AAV.ANO5 gene therapy vector was able to partially rescue several muscle deficits in *Ano5* mutants. Here, we will report gender-specific impacts of intramuscular AAV.ANO5 delivery, including citrate synthase activity and fiber type distribution. We are currently evaluating the gender-specific *Ano5* expression, as well as additional gender-biased responses to AAV. ANO5 therapy. We conclude that our mouse *Ano5* model of LGMD2L provides avenues to explore the mechanisms underlying gender bias in the human disease. Further, this work gives insight into the interplay of AAV.ANO5 gene therapy and deficits specific to gender.

268. Improved Muscle Histology in Dystrophic Mice by Exposure to Healthy Wild-Type Peripheral Circulation

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Duchenne muscular dystrophy (DMD) is a progressive muscle disease characterized by mutations in the X-linked dystrophin. Transplantation of muscle progenitor cells (MPCs) from healthy donors for treatment of DMD has been widely investigated. However, its application is hindered by poor cell engraftment caused by limited cell survival rates and immune rejection, which are most likely due to the harmful dystrophic microenvironment. Little has been done to change the muscle microenvironment in DMD patients as a therapeutic approach for enhancing the outcome of stem cell therapy and rescuing the stem cell dysfunction observed in dystrophic muscle. The parabiotic approach has been useful in revealing the striking ability of circulating factors in young mice to rejuvenate the muscle regenerative potential in old animals. In this study, we preformed parabiosis between *mdx* mice (mouse model of DMD) and young, age-matched wild-type (WT) mice having a green fluorescent protein (WT-GFP) reporter gene in order to track the circulating cells. We investigated whether muscle histopathology can be improved by using a parabiotic system to enable a constant exchange of WT peripheral blood through the skin microcirculation. Three-month-old *mdx* mice were paired with 3-month-old WT-GFP mice as the experimental group. As controls, two 3-month-old *mdx* mice were paired and two 3-month-old WT-GFP mice were paired. Mice were sacrificed at 8 weeks after parabiosis surgery. Blood smears were performed to confirm the two mice shared a network of blood vessels. Muscles which included the gastrocnemius and diaphragms were harvested, flash frozen, and cryosectioned. Hematoxylin and eosin (H&E) and trichrome staining were performed for histology analysis. Mouse IgG (marker for necrotic fiber) and F4/80 (marker for macrophage) were stained to examine inflammation. First, we found WT-GFP cells in the blood of *mdx* mice, indicating that peripheral blood cross-circulation and redistribution of circulating cells were established between the parabiotic mice in the experimental group. H&E results indicated that skeletal muscles were greatly improved in their histopathological appearance in the *mdx* mice paired with WT-GFP mice when compared to *mdx* mice paired with *mdx* mice. Trichrome and immune staining results showed decreased fibrosis and inflammation in the *mdx* mice of the experimental group compared to the *mdx* control mice. These results provide evidence that suggests the defect in MPCs in DMD is driven by the dystrophic microenvironment and might, in part, be attributable to changes in blood-borne factors, and the histological improvement observed may be related to a beneficial effect imparted on the dystrophic MPCs due to circulating factors in the blood from the healthy WT mice. These observations suggest that changing the dystrophic microenvironment may be a new approach to improve muscle weakness in DMD patients, despite the continued lack of dystrophin expression.

269. The Acta1 D286G Mouse Model of Nemaline Myopathy Displays Structural and Functional Abnormalities of Mitochondria

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Nemaline myopathy (NM) is a clinically and genetically heterogeneous muscle disorder that can cause death or lifelong disability. No effective treatments are available for NM, and therapeutic testing and development is currently hampered by a poor understanding of the disease processes involved. Clinical heterogeneity in NM is the consequence of both the genetic mutation and variations in the biological response to this mutation. Differences in disease pathology, metabolic function, and treatment response are not predictable based on our current knowledge of their causative mutation or the severity of weakness. This suggests that additional biological processes may be critically relevant to disease phenotype in NM. Preliminary protein expression analyses have implicated biological processes including mitochondrial dysfunction as potential mechanisms of weakness in NM. Of the three NM mouse models in tested in our laboratory thus far, follow-up studies have confirmed abnormalities of mitochondrial function and protein expression in Acta1 D286G mice. Overall activity of complex III in the Acta1 D286G mice was decreased, but protein expression of the complex was normal. Conversely, expression of complex IV was decreased, but normal activity of complex IV was observed on biochemical testing. Mitochondrial structure also appears

to be abnormal in some NM mouse models and in some cases of human NM. Additional classification of mitochondrial abnormalities in Acta1 D286G mice is in progress. While it is likely that these mitochondrial abnormalities are secondary to the primary contractile abnormality in NM, they also may represent a potentially treatable cause of muscle dysfunction and an exciting new direction of investigation in this disease.

Neurologic Diseases (Including Ophthalmic and Auditory Diseases) I

270. Gene Therapy for AADC Deficiency Results in *De Novo* Dopamine Production and Supports Durable Improvement in Major Motor Milestones

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Aromatic L-amino acid decarboxylase (AADC) deficiency is a rare, genetic disorder of neurotransmitter synthesis in children. AADC is responsible for the production of dopamine by a decarboxylation of the precursor L-Dopa. Dopamine is a key neurotransmitter of the striatum that supports motor function. Children with severe AADC deficiency fail to achieve motor milestones. AGIL-AADC is a recombinant, AAV2 vector containing the human cDNA encoding the AADC enzyme. We have treated 25 children with AADC deficiency using a single administration of AGIL-AADC delivered bilaterally to the putamen by stereotactic infusions during a single, operative session in single-arm, open label clinical studies at National Taiwan University Children Hospital. Patients received a total dose of either $1.8x10^{11}$ vg total of AGIL-AADC (n=21) or $2.4x10^{11}$ vg total of AGIL-AADC (n=4). Of the 25 children given AGIL-AADC, 3 are now more than seven-years post-gene therapy, 7 are more than six-years post-gene therapy, and 16 are more than two-years post-gene therapy. Clinical results of the first 18 patients given AGIL-AADC were compared to natural history cohort. At baseline, ages ranged from 21 months to 8.5 years and no child had developed full head control, sitting unassisted or standing capability, consistent with the published natural history cohort of severe AADC patients who never achieve these motor milestones over their lifetime. Compared to the natural history cohort, among the 18 patients receiving the $1.8x10^{11}$ vg total dose of AGIL-AADC, 5/15 gain full head control ($p<0.0001$), 4/15 gain sitting unassisted ($p=0.0004$), and one subject achieved standing with support at 2 years. At five years, 4/7 gain full head control and sit unassisted ($p<0.0001$), and 2/7 stand with support ($p=0.0054$). Regarding ambulatory function, two patients are using wheeled walkers, one additional patient is able to take steps holding an examiner's hand and one patient is walking independently. Adverse events in the first year after AGIL-AADC administration, in general, were associated with overall disease state. These findings indicate that gene therapy with AGIL-AADC is a potential therapeutic for patients with AADC deficiency to achieve and maintain motor milestones.

271. Intraoperative MRI-Guided Delivery of AAV2-hAADC for Parkinson's Disease: Role of Volume of Infusion in Putaminal Gene Expression

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Neurological gene delivery via direct parenchymal injections of adeno-associated viral (AAV) vectors is a locally administered treatment that requires accurate delivery to maximize safety and efficacy. The large volume and complex architecture of the human brain is a considerable barrier to translating small animal findings into efficacious clinical procedures. Inadequate distribution of the vector within the target structure may result in ineffective treatment. Conversely, excessive distribution or off-target gene delivery increases the possibility for unexpected adverse effects. To address these challenges to optimal viral vector delivery into the brain parenchyma and to minimize uncertainty related to viral vector infusions, we developed a delivery system that permits direct MRI monitoring of vector distribution within the brain in real-time by adding a MRI contrast agent to the infusate. This significant advance permitted us, for the first time in humans, to adjust parameters of vector infusion while delivering gene therapy in real-time, giving surgeons full control over gene transfer technology. Our MRI-guided delivery has been utilized in Phase 1b clinical trials in Parkinson's disease (PD), in which AAV2 containing the human aromatic L-amino acid decarboxylase gene (AAV2-hAADC) has been administered into the putamen bilaterally in 22 subjects thus far. The infusion strategy has evolved in a step-wise process that includes increasing coverage of the putamen by modifying the cannula design, increasing the infusion volume, and changing both the approach trajectories and the strategy for advancing the cannula tip through the putamen. Mean putamen coverage in the initial cohort (5 subjects per cohort; 450 µL per putamen) was 21%, increasing to 34% and 42% coverage in the second and third cohorts respectively (up to 900 µL per putamen). To further optimize delivery, an additional 7 subjects were infused with up to 1500 µL per putamen resulting in 54% mean putamen coverage. In this last cohort, infusions were performed via a trans-parietal approach with one cannula placement per putamen, whereas earlier infusions were performed via a trans-frontal approach with 2-3 cannula placements per putamen. Early evaluations have shown clinical improvements and increased [^{18}F]-DOPA binding in the putamen that correlate with the extent of putamen coverage achieved during infusion of the AAV2-hAADC vector. In summary, we found that large volumes of infusate are required to provide adequate coverage of the putamen in PD patients. These results suggest that the

failure of previous PD gene therapy trials to achieve significant clinical improvement was likely a function of insufficient delivery of viral vector (40-150 µL vector per putamen).

272. Treatment of Herpetic Keratitis with CRISPR/Cas9 Gene Editing in a Rabbit Disease Model

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Herpes Simplex Virus (HSV) ocular keratitis is an infectious disease that manifests in the corneas of HSV infected patients. HSV establishes viral latency in the neuron cell bodies within the trigeminal ganglia (TG). Reactivation of latent HSV infection can lead to recurrent episodes of herpes keratitis over time, causing corneal scarring, neovascularization, endothelial dysfunction, and vision loss. Current standard of care can ameliorate symptoms, but only reduces HSV recurrence by 50% in patients with the most severe forms of the disease. Herein, we describe a gene editing approach using CRISPR/Cas9 to target essential viral genes in order to inhibit HSV-1 replication and reactivation and, therefore, address this unmet medical need. Guide RNA (gRNA) sequences targeting essential HSV-1 genes were identified using a cell-based productive infection assay. Two gRNA sequences were selected and packaged in AAV vectors expressing CMV-driven *Staph. aureus* Cas9 (SaCas9) for follow-up studies. An efficacy study was conducted in the HSV-1 reactivation rabbit model to evaluate the potency of the CRISPR/Cas9 system with selected gRNAs. Briefly, HSV-1 strain 17Syn+ was applied to the corneas of New Zealand White rabbits post-corneal abrasion to establish viral latency in the TG. Four weeks later, rabbits were dosed with oral acyclovir (100 mg/kg, BID) as a positive control, or 1.0E+11 vg/eye of AAV-CRISPR vectors targeting RL2 or UL48, alone or in combination, or GFP (negative control). Following reactivation of latent HSV infection using epinephrine iontophoresis, viral plaque assays demonstrated a reduction in HSV-1 virions in tears by up to 64% by individual AAV-CRISPR vectors and by 75% ($p=0.03$, one-way ANOVA) in combination. Corneal lesions were suppressed in rabbits treated with individual AAV-CRISPR vectors up to 56% or 91% ($p<0.01$, one-way ANOVA) in combination. NGS-based analysis was used to determine the effects of CRISPR/Cas9 editing on latent viral genomes and INDEL distribution. The vector biodistribution, Cas9/gRNA expression profiles, and HSV viral load in both corneal and TG tissues in rabbit, as well as in murine models, are being explored to further optimize gene editing and establish the PK/PD relationship of the drug candidates. In conclusion, the current report highlights the utility of the CRISPR/Cas9 gene editing system for treating herpes ocular keratitis. Optimizing the delivery of CRISPR/Cas9 system to TG in both murine and rabbit models will also be reported.

273. Moving towards the Clinic: Intrathecal AAV9-SOD1-shRNA Administration for Amyotrophic Lateral Sclerosis

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Amyotrophic Lateral Sclerosis (ALS) is an adult-onset neurodegenerative disease, characterized by loss of motor neurons, progressive paralysis and death. Dominant mutations in the superoxide dismutase 1 (*SOD1*) gene are among the most frequent causes of inherited ALS. In our previous studies, we evaluated gene replacement therapy utilizing adeno-associated virus serotype 9 (AAV9), a vector that efficiently crosses the blood brain barrier, carrying a GFP reporter to deliver a short hairpin RNA (shRNA) downregulating SOD1. This vector significantly improved disease outcome in ALS mouse models. The treatment was well-tolerated, but the presence of a foreign transgene and regulatory elements excluded a direct application of this vector in human clinical trials. Hence, the main objective of the current study was to design and test the efficacy of an AAV9-SOD1-shRNA vector devoid of any foreign transgenes, enabling its direct use in clinical trials. We determined the *in vitro* efficiency of SOD1 downregulation and *in vivo* efficacy to delay the disease onset and progression with the modified AAV9 vector carrying a SOD1 shRNA expression cassette and a non-coding stuffer sequence instead of GFP. In mice, we tested two different delivery routes for vector administration: intravenous (IV) and intracerebroventricular (ICV). Male and female SOD1G93A mice, overexpressing human mutant SOD1, were administered the modified AAV9-SOD1-shRNA vector via IV or ICV administration at postnatal day 1 and monitored throughout their lifespan. Both treatments resulted in significant improvement in motor function tests such as rotarod performance and hindlimb grip strength. Moreover, removal of the GFP cassette markedly improved the therapeutic effect of the vector by significantly extending the median survival of ALS mice by 70 days, which is almost twice the effect achieved with the previous construct. Importantly, a similar therapeutic effect was achieved by ICV delivery of the vector at a 10-fold lower dose as compared to IV administration. To translate this approach to the clinic, we also administered the AAV9-SOD1-shRNA vector in adult (10-year-old) non-human primates (Rhesus monkeys). A single intra-lumbar infusion of the vector resulted in significant SOD1 reduction throughout the monkey brain and spinal cord, thus corroborating our dosing rationale and route of delivery. In summary, we have successfully desgined and have completed testing the modified AAV9-DOS1-shRNA vector in preclinical models as we prepared for future testing in human clinical trials. We report one of the longest survival extensions achieved in the most severe ALS mouse model, using just a single cerebrospinal fluid (CSF) administration of AAV9-SOD1-shRNA. Finally, successful translation of this therapy in non-human primates is an important step on the pathway to future human clinical trials for ALS patients.

274. The Ability of PHP.B Vector to Cross the Blood-Brain Barrier is Linked to a Genetic Trait Restricted to C57BL/6J Mice

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Identification of AAV capsids from natural isolates with central nervous system (CNS) tropism, such as AAV9, has substantially enhanced therapeutic applications of gene therapy for CNS diseases. While AAV9 is unique in terms of CNS delivery, its efficiency to transduce the CNS following systemic administration is limited to newborns. Deverman *et al.* recently described an engineered variant of AAV9, called PHP.B, that exhibits much higher delivery to the CNS of adult mice following intravenous (IV) injection. A Cre transgenic line in C57BL/6J background was used to select this capsid variant based on efficient trafficking to the CNS. In an attempt to characterize the translational potential of the PHP.B capsid, we evaluated its efficacy in different strains of mice as well as in nonhuman primates (rhesus macaque). Prior to doing so, we confirmed the findings reported by others in C57BL/6J mice. IV injection of 1E12 GC of PHP.B expressing GFP demonstrated remarkable transduction throughout the brain and the spinal cord, as measured by detection of GFP fluorescence, that was almost 4 logs higher than what was observed with AAV9. Surprisingly, PHP.B injection in BALB/cJ mice at the same dose produced very low brain transduction with vector genome copies 2 logs lower in the CNS of BALB/cJ compared to C57BL/6J mice. Injecting 1E11 GC of the same vectors via intracerebroventricular administration, we observed high brain transduction in both C57BL/6J and BALB/cJ mice, demonstrating that blood-brain barrier penetration, and not neurotropism, is affected by the strain of mice. Despite the disappointing results obtained from IV injection in BALB/cJ mice, we proceeded with pilot studies in NHPs. Adult rhesus macaques (N=1 per vector) that were negative for neutralizing antibodies to the respective capsids were injected with 2E13 GC/kg or 7.5E13 GC/kg IV of the same GFP versions of AAV9 and PHP.B studied in mice. Both NHPs tolerated the lowest vector dose well, with only minor abnormalities in clinical pathology, whereas at the higher dose of 7.5E13 GC/kg the animal injected with the PHP.B vector developed acute toxicity that required euthanasia on day 5 (toxicity event described in another abstract). GFP expression was detected by direct fluorescence and quantified in most non-CNS tissues, including liver, muscle, kidney, pancreas, heart, spleen, and pituitary at comparable levels in the AAV9 and PHP.B-treated animals (except for some discrepancies due to early necropsy in the high-dose PHP.B). However, very low transduction was observed with both capsids in the CNS based on GFP fluorescence. Interestingly, the dorsal root ganglia, which lie outside of the blood-brain barrier, were transduced at equally high levels with both AAV9 and PHP.B. Evaluation of vector genomes in tissues tracked with GFP expression; notably, vector genomes were 3 logs lower in tissues of the spinal cord and brain than in liver and spleen, with no differences between AAV9 and PHP.B. *In vivo* transduction of vectors based on natural isolates of AAV has generally tracked between strains of mice and different species, with the notable exception of liver where mice are more efficiently transduced than primates or dogs. The apparent restriction of PHP.B properties to C57BL/6J mice may reflect the fact that it was selected in this genetic background of mice. Ongoing studies using C57BL/6J and BALB/cJ F1 and F2 hybrids show a Mendelian inheritance profile with 100% of F1 displaying intermediate CNS transduction; while the F2 generation shows a distribution of transduction with 50% intermediate, 25% high, and 25% low CNS transduction. Mouse whole exome sequencing analysis is currently ongoing to identify the allele(s) linked with blood-brain barrier penetration in mice.

275. Subfoveal Injection without Vitrectomy of a Photoreceptor-Targeted AAV5 is Both Safe and Efficacious in Cynomolgus Macaques

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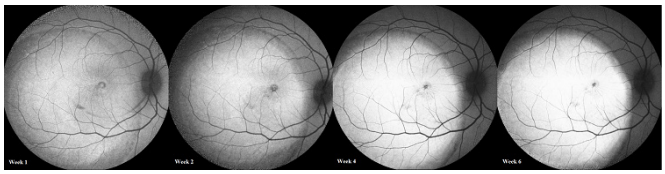
Purpose: Subfoveal delivery of AAV has become standard of care in some gene therapy clinical trials, with vitrectomy performed prior to vector injection. We proposed that subfoveal injections could be successfully performed in primates with maintenance of an intact vitreous humor. **Methods:** Four cynomolgus macaques were injected bilaterally with AAV5 containing the hGRK1 promoter driving GFP at a concentration of 1e12 vg/ml. A three-step injection was utilized, with a small volume of fluid used to induce foveal detachment, followed by aqueous paracentesis, then vector solution injection to expand the subretinal bleb. In two animals, vehicle was used to induce foveal detachment, then 100µL of AAV5 for bleb enlargement (Group 1). In the other two animals, a total of 100µL AAV5 was used for both steps (Group 2). Serum neutralizing antibodies (Nab) were analyzed pretest and at study termination 6 weeks post-injection. Ophthalmic examinations, optical coherence tomography (OCT), and confocal scanning laser ophthalmoscopy (cSLO) were performed at regular intervals. **Results:** Subfoveal delivery of AAV5 was accomplished in all 8 eyes. One Group 2 eye only received 50µL AAV5 after development of apparent retinal tension during injection. One Group 1 eye developed a macular hole after an air bubble was inadvertently introduced during bleb enlargement. The macular hole showed resolution on OCT after 1 week, but a subfoveal detachment persisted through study termination. Remaining eyes had resolution of detachment at 1 week, and return of the ellipsoid zone and foveal bulge in 5/7 eyes by study termination. cSLO revealed faint GFP fluorescence at 1 week, becoming progressively stronger through 6 weeks. Mild uveitis was evident in the animal with a macular hole at 1 week post-injection, but resolved by week 2 with anti-inflammatory treatment. Both Group 2 animals developed minimal intermediate uveitis in both eyes from weeks 1 through 6. NAb titers were negative in all animals pretest. One Group 2 animal developed a strong anti-AAV5 response at 6 weeks, while responses in all other animals were modest. **Conclusions:** This three-step subfoveal injection without vitrectomy is effective for delivery of AAV in normal macaques. Use of vehicle instead of AAV vector solution to create the initial foveal detachment may decrease subsequent vitreal inflammation, and may have an impact on NAb formation.



276. Rescue of Amyloid Deposition Phenotype after Single-Treatment CRISPR/Cas9 Gene Editing in a Humanized Mouse Model of TTR Amyloidosis

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Introduction: Transthyretin amyloidosis (ATTR) is a systemic, debilitating and fatal disease caused by accumulation of amyloid deposits of the transthyretin (TTR) protein in multiple tissues. The majority of TTR protein is produced by and secreted from the liver, and reduction of hepatic TTR protein is a clinically-validated approach to ATTR disease management. Several TTR-lowering drugs are in development, sharing a common mechanism of targeting *TTR* mRNA for destruction. These drugs will likely require chronic dosing over a patient's lifetime to maintain clinical benefit, due to the transient nature of the suppression. A more convenient approach for this patient population would be a permanent reduction of TTR expression, such as could potentially be provided by CRISPR/Cas9 gene editing with a single or limited number of treatments. **Methods**: To explore the feasibility of sustained reduction of TTR expression in an ATTR disease model, we developed lipid nanoparticle (LNP) formulations containing a single chemically modified guide RNA specifically targeting the human TTR gene along with an mRNA encoding the *S. pyogenes* Cas9 nuclease. These formulations were evaluated in a well-established mouse model of hereditary ATTR amyloidosis that expresses the V30M pathogenic mutant form of human TTR protein and exhibits deposition of TTR within multiple tissues over time. **Results**: Our results demonstrate that a single administration of LNP led to durable and substantial reduction of TTR mRNA and protein expression in the liver, with a concomitant reduction in circulating serum TTR protein levels. Sustained reduction of hepatic TTR expression over a two-month period correlated with a marked reduction of TTR protein deposition in pathologically relevant tissues within the peripheral nervous system and GI tract. No changes in animal behavior or health were observed throughout the course of the study. **Conclusions**: These findings highlight the potential of *in vivo* CRISPR/Cas9 gene editing and suggest that future therapies based on this platform may enable next-generation acute treatment paradigms for chronic diseases such as ATTR.

277. Gene Therapy for *Glut1*-Deficient Mouse Using AAV Vector with the Human Intrinsic Glut1 Promoter

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Background: Glucose transporter 1 deficiency syndrome (GLUT1DS, OMIM #606777) is an autosomal dominant disorder caused by haploinsufficiency of *SLC2A1*, the gene encoding GLUT1. Heterozygous mutation of *SLC2A1* results in impaired hexose transport into the brain and finally irreversible neurologic disorders. Previously, we generated an AAV9/3 vector in which *SLC2A1* was expressed under synapsin I promoter (AAV-h*SLC2A1*), and suggested that AAV-h*SLC2A1* administration improved motor function of heterozygous knock-out murine *Glut1* ($GLUT1^{+/-}$) mice. This time, we developed another AAV vector that approximates human physiological GLUT1 expression with the human intrinsic GLUT1 promoter. **Methods:** We extrapolated human endogenous GLUT1 promoter sequences that situates 5' UTR indicating high homology with rat Glut1 promoter sequences that was associated with basal transcription. We generated the tyrosine-mutant AAV9/3 vector in which human *SLC2A1-myc-DDK* was expressed under the assumed human GLUT1 promoter (AAV-GLUT1). AAV-GLUT1 was injected into $GLUT1^{+/-}$ mice with intra-cerebroventricular injection (CNS-local; 1.85×10^{10} vg/mouse or 6.5×10^{10} vg/mouse). We analyzed GLUT1 mRNA/protein expression in the brain and other major organs, cerebral microvasculature by lectin-staining, motor function using rota-rod and footprint tests, and blood and cerebrospinal fluid (CSF) glucose levels. Additionally, we confirmed exogenous GLUT1 protein distribution in the brain and other organs after intra-cardiac injection (7.8×10^{11} vg/mouse). **Results:** Exogenous GLUT1 mRNA was detected in the cerebrum in both AAV-GLUT1 doses. GLUT1 protein was mainly expressed in endothelial cells, and partially expressed in neural cells and oligodendrocytes, and strongly expressed in whole cerebral cortex. Exogenous GLUT1 expression was maintained in the cerebral cortex, hippocampus, and thalamus for 6 months. Cerebral microvasculature was increased compared to un-injected control $GLUT1^{+/-}$ mice. The motor function test and CSF glucose levels were significantly improved. Exogenous GLUT1 was not detected in the outside CNS after intra-cerebroventricular injection, and detected in the liver and muscle tissue after intra-cardiac injection of AAV-GLUT1. **Conclusions:** AAV-GLUT1 injection generated exogenous GLUT1 expression which was similar to intrinsic GLUT1. The cerebroventricular injection of AAV-GLUT1 improved CSF

glucose levels and motor function of *GLUT1* -deficient mice with no off-target distribution. Exogenous GLUT1 expression on endothelial cells remained more than 6 months.

278. Novel Gene Therapy Approaches for Whole Brain Delivery of the Lysosomal Gcase Enzyme for Wide Protection from Alpha-Synuclein Toxic Aggregates

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Alpha-synuclein (alpha-Syn) toxic aggregates accumulate over time throughout large brain areas in Parkinson's disease (PD), MSA and Lewy body dementia and are responsible for cortical functional decline leading to severe cognitive deficits. Approximately 5-8% of PD patients are carriers of a heterozygous GBA1 mutation, causing a detectable reduction in GCase global activity. Conversely, stimulating GCase activity has been shown to fuel lysosomal activity to stimulate degradation of alpha-Syn deposits. We have established two novel gene therapy approaches to widely express GCase in the whole brain and, thereby, reaching a global protection from alpha-Syn-dependent dysfunctions. First, we showed that a single intravenous injection of the brain penetrant GBA1 expressing AAV-PHP.B is sufficient to provide robust and long-lasting protection from alpha-Syn deposits in a mouse model of synucleinopathy. AAV-PHP.B delivered GCase is targeted to the lysosome and acquires functionality, which resulted in significantly diminished accumulation of insoluble alpha-Syn species in all the forebrain regions. However, it remains to be shown whether this AAV serotype will maintain the capability to efficiently permeate the blood-brain barrier in humans. Alternatively, we conceived a different approach based on the cell-to-cell spreading of the GCase enzyme. In fact, we showed that GCase can be released by brain endothelial cells and reuptaken by surrounding neurons and astrocytes. Thus, we generated GBA1-expressing AAV-BR1 particles able to specifically target the brain microvasculature and allow GCase to diffuse throughout the central nervous system. Remarkably, hA53T-alpha-Syn transgenic mice subjected to this treatment exhibited a widespread reduction of alpha-Syn deposits in the cerebral cortex. We propose that the AAV-BR1-based gene therapy is a crucial strategy to convert the brain microvasculature in a stable source of supplemental GCase enzyme for the long-term protection of neural tissue from accumulating alpha-Syn aggregates.

279. Phase I Trial Implanting Peripheral Nerve Grafts into the Substantia Nigra in Patient's with Parkinson's Disease at the Time of Deep Brain Stimulation Surgery

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Over the last five years, we have been evaluating the safety and feasibility of investigating a cell therapy delivered to the substantia nigra in participants (n=38) with Parkinson's disease as part of Phase I open-label, single center, clinical trials (NCT01833364 and NCT02369003). Our novel approach combines surgical deployment of the investigational tissue with deep brain stimulation (DBS), which we have termed, DBS Plus. A major ethical advantage of DBS Plus is that participants do not have to forego the therapeutic benefits of DBS to be involved in the study. The source of our cell therapy material is autologous peripheral nerve tissue obtained from the sural nerve. Schwann cells are abundant in peripheral nerve tissue and transdifferentiate after injury into "repair cells". Tissue grafts are harvested and implanted into the substantia nigra as part of the same surgical procedure during DBS surgery directly following the placement of the stimulating electrodes. Using an adaptive trial design, we have been able to evaluate the safety and feasibility of different aspects and dosages of deploying grafts. Most recently, as part of a 2-year long follow-up, we implanted 17 participants with peripheral nerve grafts to the substantia nigra and DBS electrodes to the globus pallidus interna. The adverse event profile has been comparable to DBS alone. For 9 participants who have reached the 2 year time point, Unified Parkinson's Disease Rating Scale (UPDRS) scores (motor scores) off DBS stimulation and off medications, for 12 hours or more, was 7.3 ± 10.5 points lower (less symptoms- considered moderate clinical improvement) than at baseline (27.7 ± 12.7 points at 2 years vs. 35.0 ± 11.4 points at baseline). Early Phase I results at 2 years after surgery support that DBS Plus is a safe and feasible platform to trial cell therapies. Meanwhile, clinical efficacy results appear to support moving to a Phase II trial to begin testing the efficacy of this investigational therapy.

280. Increase in Primary Visual Cortex Dendritic Complexity Prompted by Retinal Gene Therapy

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Introduction: Retinal diseases cause progressive deterioration of vision leading to complete visual deprivation. As reported in prior animal studies, visual deprivation causes dramatic changes in dendritic population of the visual cortex, decreasing the dendritic branching with eyelid sutured animals and increasing the complexity of dendrites when sutures were reopened. Whether similar processes take place in human

blindness upon vision restoration has never been previously studied. However, recent advances in diffusion magnetic resonance imaging (dMRI) makes such a study possible through assessment of the density and angular variation of dendrites *in vivo*. Having access to a group of Leber's congenital amaurosis (LCA) patients who underwent bilateral retinal gene therapy (GT) to regain their vision allows for parallels to be drawn between human and animal studies. The objective of this study was to evaluate the effect of GT on the complexity of dendritic trees in the primary visual cortex of LCA patients using an advanced dMRI method called neurite orientation dispersion and density imaging (NODDI). **Methods:** Ten patients with LCA underwent dMRI imaging at baseline, 1, 3, 6 months, and 1 year after GT as well as ten demographically matched normal sighted controls. Diffusion weighted images were motion and eddy-current corrected using FMRIB Software Library (FSL) and then fit to the NODDI model using the NODDI Toolbox in MATLAB. Single subject templates of T1-weighted images (T1) were created using the Advanced Normalization Tools (ANTS) software package. Next, b=0 dMRI images were aligned to the single subject T1 templates. Calcarine fissure region of interests (ROIs) were drawn on the single subject T1 atlas using ITK-Snap software and then dilated to include surrounding grey matter. Voxels that had high isotropic diffusion in the NODDI images were added to the fissure segmentation label to exclude partial volume effects in the cortical ROIs. Lastly, a random effect repeated measures statistical analysis was performed comparing changes in the orientation dispersion index (ODI) and cerebral spinal fluid (CSF) content over time. **Results:** The restoration of vision through GT caused transient but significant ($P = 0.021$) increases in the ODI of the inferior calcarine cortex indicating an increase in dendritic complexity. The increase in tissue complexity was mainly observed for the right inferior calcarine cortex with the maximum amplitude of change occurring at 3 months after GT (0.553 to 0.575, $P = 0.043$). Changes in dendritic complexity for the superior calcarine cortex did not reach statistical significance ($P > 0.16$). No statistically significant changes were observed over time for the calcarine dendritic complexity in controls. Interestingly, significant decreases in the CSF content within the calcarine fissure were observed, implying growth of other tissue. This negatively correlated with changes in dendritic complexity in the cortex (dip at 3 months (0.401 to 0.316, $P = 0.0033$)). **Conclusion:** The NODDI method allows for evaluation of changes in the tissue complexity of the primary visual cortex that may occur as a result of reinstating vision. Previously, such data was only available in animal models for vision deprivation or restoration. Here, for the first time, in parallel with prior animal studies, changes in dendritic complexity of the primary visual cortex tissue are demonstrated in a group of LCA2 patients who regained binocular vision through GT. Thus, retinal gene therapy not only recovers retinal function, but promotes a profound downstream transformation within the primary visual cortex to improve vision.

281. Enhanced Version of Human Sulfamidase Significantly Ameliorates CNS Pathology When Delivered to the MPS-IIIA Mice by AAV-Mediated Intra-CSF Injection

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Mucopolysaccharidosis type IIIA (MPS-IIIA) is one of the most common and severe forms of neurodegenerative lysosomal storage disorders (LSDs). MPS-IIIA is caused by inherited defect of the lysosomal hydrolase Sulfamidase (SGSH) that leads to the accumulation of toxic material into the cells. The central nervous system (CNS) represents the most affected tissue in MPS-IIIA. The main goal of our laboratory is to develop AAV-mediated gene transfer approaches to treat the CNS in LSDs with minimal invasiveness and high CNS targeting efficiency. Intrathecal (IT) injection into the cerebrospinal fluid is an attractive administration route to deliver therapeutic genes to the CNS with potentially minimal invasiveness. We recently characterized the CNS transduction pattern of several AAV serotypes upon intra cerebral spinal fluid (CSF) administration in a large animal model (WT pigs). We therefore developed and tested an intra-CSF AAV-mediated gene transfer approach for MPS-IIIA based on the use of the most performing AAV serotype bearing a modified version of human Sulfamidase enhanced in terms of secretion and activity (E-hSGSH) compared to the wild type one. In order to test the functionality of the enhanced version of hSGSH we injected intra-CSF both WT pig and MPS-IIIA mice with AAV vectors expressing the E-hSGSH. Significant increase in the hSGSH activity and amount of protein into the brain and liver confirmed the effectiveness of E-hSGSH in both large and small animal models. Moreover, E-hSGSH was also able to both rescue brain storage pathology and improve behavioural performance in adult MPS-IIIA mice. These data provide a proof of principle demonstrating that the enhanced version of Sulfamidase represents a therapeutic potential for the design of a low-invasive strategy to treat the brain and also systemic pathology in MPS-IIIA.

282. AAV-Mediated Gene Delivery to Treat Xeroderma Pigmentosum - Cockayne Syndrome (XP-CS) *In Vivo*

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Introduction Cockayne Syndrome (CS) is a rare, autosomal recessive disorder characterized by neurodegeneration and premature aging. CS

is caused by mutations in genes involved in DNA repair mechanisms. One of these mutations affects the XPG protein, an endonuclease required for nucleotide excision repair, which is encoded by the ERCC5 gene. Patients with mutations in ERCC5 present with UV sensitivity, and may also display developmental defects, and various neurological issues, with symptom alleviation being the only available treatment. Adeno-associated virus (AAV) is a highly promising candidate for CS gene therapy due to its safety, as well as high level and long-term gene expression. The aim of this project is to develop a gene therapy for CS and study its impact upon progression of the disease in a knockout-based mouse model following intravenous injections of AAV9-CMV-coERCC5 vector as compared to healthy (WT) and untreated ($XPG^{-/-}$) controls. Hypothesis We hypothesize XPG gene delivery will provide some degree of correction of the neurodegeneration and premature aging observed in $XPG^{-/-}$ mice. Methods $XPG^{-/-}$ mouse pups (1-2 days of age) were intravenously administered $5x10^{12}$ vector genomes/kg (n=3) or $1x10^{13}$ vg/kg (n=10). Untreated $XPG^{-/-}$, treated $XPG^{-/-}$, and WT mice were evaluated weekly by neurological (surface righting, negative geotaxis, and clasping), physical (weight and length), and behavioral (distance, resting time, slow movement, fast movement, and rearing) examinations for up to 30 weeks. Mice were euthanized upon reaching a moribund state or at 30 weeks and complete necropsies were performed for further analysis. Gene expression was evaluated using quadricep muscles and Real Time - Polymerase Chain Reaction (RT-PCR).Results Studies are ongoing. Data acquired thus far show improvements in several neurological exams and activity tests for all AAV-treated mice. RT-PCR revealed the upregulation of XPG gene expression in both AAV-treated groups as compared to untreated $XPG^{-/-}$ controls. Future Directions An additional cohort of $XPG^{-/-}$ mouse pups (1-2 days of age) will be intravenously administered therapy at $5x10^{13}$ vector genomes/kg. Further analyses are in progress to evaluate the extent of improvement from gene therapy for CS. Future investigations will include histology studies on collected tissues, as well as further gene, protein, and functional analyses.

283. AKT3 Gene Transfer Promotes Photoreceptor Neuroprotection in a Pre-Clinical Model of Retinitis Pigmentosa

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Retinitis pigmentosa (RP) is a collection of inherited retinal dystrophies affecting an estimated 1:4000 individuals globally. Clinical onset is characterized by impairments in night vision coinciding with the death of rod photoreceptors. As this process expands, it destroys peripheral vision and culminates in a condition of severe vision loss mediated by the progressive degeneration of cone photoreceptors, which are required for daylight, high acuity, and color vision. While gene replacement therapy is a promising therapeutic approach, the genetic heterogeneity of this disease and limitations of conventional gene transfer vectors present formidable challenges to develop a treatment for each specific disease-causing gene. A recently described mechanism of photoreceptor degeneration in RP involves insufficient nutrient uptake/utilization associated with downregulation in anabolic metabolism, particularly pathways controlled by mTOR signaling activity. Here we interrogated the effects of anabolic reprogramming on photoreceptor survival and function in a pre-clinical model of RP using a conventional gene augmentation strategy. Specifically, we hypothesized that stimulation of anabolic metabolism via overexpression of the serine/threonine kinase AKT would promote photoreceptor neuroprotection and possibly preserve visual function in the *Pde6b*^{rd10} mouse retina. We generated AAV vectors that drive ubiquitous expression of human *AKT3* fused to an N-terminal myristoylation signal. Following subretinal delivery of AAV vectors, animals were evaluated at two time points with electroretinography and optokinetic response to assess visual function. Unfortunately, we did not observe statistically meaningful differences in functional preservation between AAV-AKT3 treatment and controls. However, histological analysis of retinal cross-sections revealed robust preservation of photoreceptor numbers and structural morphology near the regions of subretinal delivery compared to non-transduced areas and control retinas that remained untreated or treated with AAV-GFP. In addition, AKT3 gene transfer stimulated mTOR complex 1 (mTORC1) activity, a signaling pathway previously shown to enhance photoreceptor survival in this disease context. Finally, we examined retinal samples for the potential of oncogenic transformation by immunostaining with established markers of cell proliferation. Staining with these markers revealed no differences between AAV-AKT3 and control treatments, suggesting this activity is indeed preservative rather than proliferative. Collectively, this investigation highlights a potential broad spectrum approach to promote photoreceptor survival during complex retinal degenerative disease.

284. AAV.PHP.B Transduction is Restricted to the Spinal Cord after Vascular Delivery in a Sheep Model of Tay-Sachs Disease

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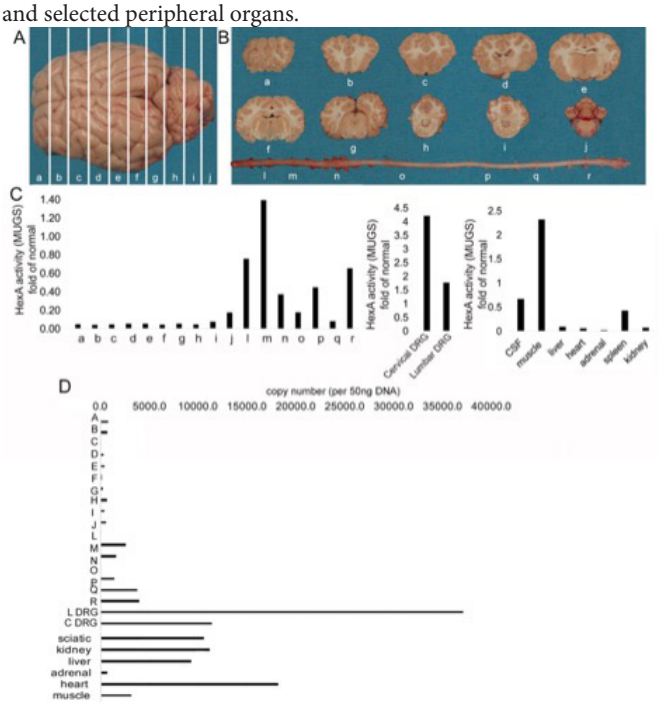
Tay-Sachs disease (TSD) is a fatal neurodegenerative disorder of children that is caused by mutations in the heterodimeric lysosomal enzyme N-acetyl-hexosaminidase A (HexA), which results in storage of GM2 ganglioside in the central nervous system (CNS). Hex A consists of α and β subunits, which are encoded by the *HEXA* and *HEXB* genes, respectively. To effectively breakdown GM2 ganglioside in humans and sheep, both the α and β subunits must be expressed in the same cell at similar levels. To ensure coexpression after intravascular delivery we tested the therapeutic efficacy of a second generation bicistronic AAV vector encoding both sheep α and β subunits. Two affected TSD sheep were injected intravenously (jugular vein) with 2E13 vg/kg of bicistronic AAV.PHP.B vector at 8 days of age. Less than three weeks after treatment one sheep developed neurologic disease which progressed to loss of the extensor carpi radialis reflex and ability to extend its forelimbs bilaterally. This sheep had to be euthanized which is approximately 6.5 months prior to the typical humane endpoint

of an untreated TSD sheep. No increase in lifespan was noted in the other AAV treated TSD sheep compared to untreated TSD controls, however gene therapy may have corrected the muscle atrophy typical of untreated TSD sheep. Ultra-high field MRI and MR spectroscopy showed neurodegeneration of the brain and spinal cord consistent with untreated TSD disease progression. Post-mortem analysis of the sheep that reached endpoint at 3 months of age revealed no significant alternations beyond that observed in age matched untreated TSD sheep. Analyses performed included immunohistochemistry for astrocytosis (Gfap) and microgliosis (Iba1), oligodendrocytes (Olig2), neurons (Map2) and myelination (luxol fast blue) as well as radial nerve pathology. HexA activity and vector genome biodistribution were measured in the central nervous system and selected peripheral tissues (Fig 1). HexA activity was increased to 20-140% or normal in the spinal cord and in the cerebrospinal fluid (CSF). Enzymatic activity in dorsal root ganglia of the cervical intumescence exceeded 4-fold over normal and no neurodegeneration was noted. HexA also increased in skeletal muscle and spleen (Fig 1). Vector genome biodistribution mimicked that of HexA activity. Overall, these data suggest that transduction of PHP.B does not exhibit widespread neuronal transduction of the brain of sheep as previously reported in mice. An atypical phenotype and premature euthanasia was observed in one of the AAV treated sheep, but a causative reason has yet to be identified.
Figure 1. Biodistribution of AAV.PHP.B vector containing a bicistronic vector encoding for both HEXA and HEXB in one AAV treated TSD sheep. A) a dorsoventral view of the sheep brain. B) Coronal sections of the brain (a-j), spinal cord (l-r) are shown indicating sections analyzed. C) HexA activity of these tissues are reported as fold of normal of the brain, spinal cord, cervical and lumbar DRGs, CSF and selected peripheral tissues. D) Quantitative PCR of the central nervous system and selected peripheral organs.



285. Characterizing the Window of Gene Therapy Treatability in M-Opsin Knock-Out Mice, a Model for Blue Cone Monochromacy

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Purpose: Blue cone monochromacy (BCM) is an X-linked congenital disorder with severe cone dysfunction due to the absence of the long and medium wavelength-sensitive cone opsins. Previously we have shown that cones in the dorsal retina of M-opsin knockout (*Opn1mw*$^{-/-}$) mice do not form outer segments, resembling cones lacking outer segments in the human BCM fovea. We also showed that AAV5-mediated expression of either human M- or L-opsin promoted regrowth of cone outer segments and rescued M-cone function in the treated *Opn1mw*$^{-/-}$ dorsal retina. In this study, we seek to determine cone viability and window of treatability in aged *Opn1mw*$^{-/-}$ mice. **Methods:** We labeled retinal whole mounts of *Opn1mw*$^{-/-}$ mice of different ages with peanut agglutinin (PNA) to assess cone viability. AAV5 vector expressing human L-opsin driven by the cone-specific PR1.7 promoter was injected subretinally into one eye of seven month old *Opn1mw*$^{-/-}$ mice, while the contralateral eyes remain untreated and served as controls. M-cone mediated retinal function was analyzed by full-field electroretinography (ERG) under middle wavelength light condition (510nm). L-opsin transgene expression and its cellular localization were examined by immunohistochemistry. **Results:** PNA binds to the extracellular glycoprotein matrix of cone sheath that is secreted by cone inner segments and is therefore a marker of cone structural viability even in the absence of cone outer segment integrity. We show that PNA staining in 7 month old *Opn1mw*$^{-/-}$ whole mounts have numbers of positive PNA-stained cells equivalent to those in age-matched wild type mice. This demonstrates that cone sheaths remain intact and that these dorsal cones remain viable at this age. Consistent with this observation, AAV5-mediated expression of human L-opsin rescues M-cone function and restores outer segment morphology when treatment is initiated at 7 month of age in *Opn1mw*$^{-/-}$ mice. **Conclusions:** Dorsal cones of *Opn1mw*$^{-/-}$ mice without visual pigments remain viable for at least 7 months and can still be functionally and structurally rescued by cone opsin gene therapy. These results have important implications for BCM patient entry selection in any future BCM gene therapy clinical trials.

286. Raav-Mediated *PEX1* Retinal Gene Augmentation Reduces Vision Loss in a Mouse Model for Mild Zellweger Spectrum Disorder

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Goal. Zellweger spectrum disorders (ZSDs) are a group of autosomal recessive disorders caused by mutations in any one of 13 PEX genes whose protein products are required for peroxisome assembly and function. More mildly affected individuals harboring the common PEX1-G843D mutation consistently develop a retinopathy that progresses to blindness. To test whether we could slow visual loss in these patients, we performed a proof-of-concept trial for PEX1 retinal gene augmentation therapy using our mouse model homozogyous for the equivalent Pex1-G844D mutation. This model exhibits a gradual decline in scotopic full field flash electroretinogram (ffERG) response, an always residual photopic ffERG response, diminished visual acuity, and photoreceptor cell anomalies. **Methods.** AAV8.CMV.hPEX1.HA vector was administered by subretinal injection to 2 mouse cohorts of 5 or 9 weeks of age; AAV8.CMV.GFP vector was used as a control in the contralateral eye. Efficient expression of the virus was confirmed by retinal histology/immunohistochemistry, and its ability to recover peroxisome import was confirmed in vitro. Preliminary ffERG and optokinetic (OKN) analyses were performed on a subset of animals at 8, 16, and 20 weeks after gene delivery. Final ffERG and OKN measures were performed when each cohort reached 32-weeks of age (23 or 27 weeks post injection). Results. Preliminary ffERG and OKN analyses at 8 weeks post injection showed mildly better retinal response and visual acuity, respectively, in the PEX1-injected eyes, as did ffERG analysis when each cohort reached 25-weeks of age (16 or 20 weeks after gene delivery). This effect was more pronounced in the cohort with intervention at 5 weeks of age, when ffERG response is highest in homozygous Pex1-G844D mice. At 32 weeks of age, the average ffERG response in the PEX1-injected eyes was double that of GFP-injected eyes in both cohorts. Furthermore, in PEX1-injected eyes the photopic ffERG response improved over time, and the decline in scotopic b-wave amplitude was ameliorated compared to GFP-injected eyes. OKN results trended towards improvement albeit non-significant. **Conclusions:** AAV8.CMV.hPEX1.HA was delivered subretinally into the left eye of 5 and 9-week-old homozygous Pex1-G844D mice. Successful protein expression with no gross histologic side effect was observed. Neither the injection, nor exposure to the AAV8 capsid or the transgenic protein negatively altered the ffERG or OKN response. At 5-6 months after gene delivery, therapeutic vector-treated eyes showed improved ffERG compared to control eyes, on average, in both the younger "prevention" and older "recovery" cohorts. This implies clinical potential of retinal gene augmentation to improve vision in patients with ZSD at both earlier and later stages of disease.

287. Second Generation AAV Vectors Ameliorate GM2 Gangliosidosis after Systemic Delivery

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Background: GM2 gangliosidoses, including Tay-Sachs disease (TSD) and Sandhoff disease (SD), are fatal lysosomal storage disorders caused by mutations in the *HEXA* and *HEXB* genes, respectively. These mutations render the β-N-hexosaminidase A (HexA) isozyme dysfunctional, leading to the accumulation of GM2 ganglioside (GM2) in neurons with ensuing neurodegeneration, and eventual death of patients by the age of 5 years. Until recently, the most successful therapeutic effects have been achieved through intracranial co-delivery of two monocistronic adeno-associated viral (AAV) vectors encoding HexA α and β-subunits in mouse and cat models of SD. While recent studies have shown neonatal systemic delivery of AAV9-HEXB vectors to be effective in treating SD mice, this approach is flawed due to a species-specific metabolic pathway that allows HexB (ββ homodimers) to participate in catabolism of GM2. In humans this pathway is non-functional and thus co-delivery of α and β-subunits is necessary to overexpress the heterodimeric (αβ) HexA protein and supply corrective levels of enzyme throughout the CNS and eliminate GM2 storage. To address this need, we developed second-generation AAV vectors carrying simultaneously *HEXA* and *HEXB* and tested their therapeutic efficacy by systemic administration in 4 week-old SD mice. Methods: This study compared the efficacy of four bicistronic AAV vectors with two different transgene designs encoding mouse proteins, referred to as bicistronic v2 and P2I, each packaged with AAV9 and AAV-PHP.B capsids. Four week-old SD mice (*HexB*$^{-/-}$) were treated systemically with one of the four vectors, n=22 (11 males, 11 females) for each vector, at a dose of 4e12 vg (n=14) and 1e12 vg (n=8) for AAV9 vectors and 1e12 vg (n=14) and 3e11 vg (n=8) for PHP.B vectors. A subset of animals treated with the high dose for each group (n=6) was sacrificed at 150 days of age, while the remaining mice were assessed for survival. PBS injected wild type and knockout mice (n=6) were used as controls. Vector efficacy was assessed by behavioral and biochemical outcome measures. Rotarod and inverted screen tests were performed at 60, 90, 105, 120, and 149 days of age for animals treated with a high dose vector. Central nervous system Hex activity and GM2 ganglioside content were measured to correlate neurochemical impact with changes in survival and behavioral performance. Results: Performance in the rotarod and inverted screen tests of AAV-treated SD mice remained comparable to normal controls, unlike untreated SD mice where performance declined rapidly over time until humane endpoint (~129 days). All AAV treated animals in the high-dose subset survived to the experimental endpoint of 150 days. Presently, all treated mice assessed for survival are between 200 and 340 days of age, with the exception of the P2I-AAV9 group. Hex activity in cerebrum and cerebellum was

increased in all AAV treatment groups, except in the cerebellum of P2I-AAV9 treated SD mice. Conclusion: This next generation of AAV vectors for GM2 gangliosidoses prevented disease onset and extended survival of SD mice treated after systemic administration in 4-week old animals. The impressive therapeutic efficacy documented with both AAV9 and AAV-PHP.B vectors supports the clinical translation of these second-generation AAV vectors for treatment of TSD and SD patients by systemic administration.

288. Intravenous Delivery of AAV Gene Therapy in GM1 Gangliosidosis

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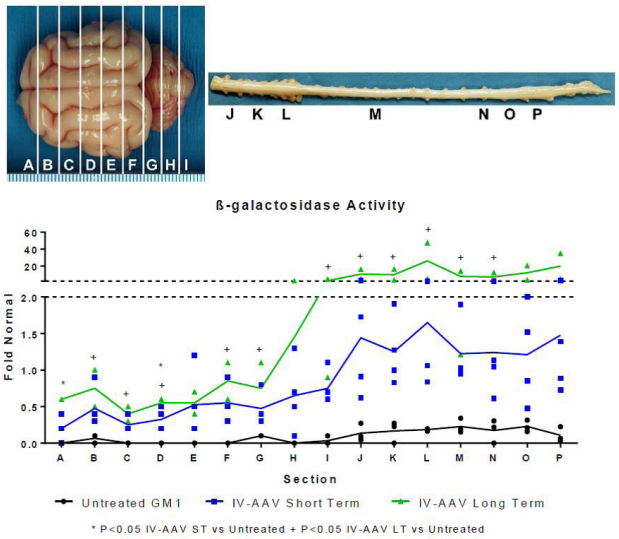
GM1 gangliosidosis is a hereditary lysosomal storage disease caused by a deficiency of lysosomal β-galactosidase (β-gal). The most common form of GM1 gangliosidosis affects children, is fatal by 4 years of age, and is characterized by rapidly progressing neurological disease. Outside of palliative and supportive care, there is no effective treatment for GM1. Adeno-associated viral (AAV) therapy has proven effective in a well-characterized feline model of GM1 gangliosidosis, demonstrating a greater than 10-fold increase in lifespan after injection to the brain thalami and deep cerebellar nuclei. Intravenous delivery was tested to circumvent the surgical risk of the intracranial approach while potentially increasing cortical and systemic biodistribution. AAV9 was delivered at a total dose of 1.5e13 vector genomes/kg body weight at approximately 1 month of age. The six animals in the study were divided into two cohorts: 1) a long-term cohort, which was followed to humane endpoint, and 2) a short-term cohort, with samples collected 16 weeks post treatment. Animals were assessed using a clinical rating score to determine disease progression. At the designated time point, biodistribution of β-gal and vector were assessed, using a synthetic enzyme substrate and qPCR, respectively. Biomarkers of disease progression were studied in the CSF, and brain metabolites were analyzed using magnetic resonance spectroscopy (MRS). The long-term group (n=2) had an average 5.3-fold increase in life expectancy, with both animals showing limited neurological signs. The short-term cohort (n=4) also showed amelioration of clinical symptoms up to 16 weeks post-treatment. In both cohorts, there was an increase in the distribution and activity of β-gal, reaching normal levels in the cerebellum, spinal cord (cervical, mid-thoracic and lumbar regions), and peripheral tissues such as the heart, skeletal muscle, and sciatic nerve. Analysis of CSF biomarkers, such as aspartate aminotransferase and lactate dehydrogenase, showed a normalization in comparison to untreated animals. Additionally, MRS metabolites such as myoinositol, N-acetyl aspartate and glycerophosphocholine / phosphocholine indicated amelioration of gliosis, improved neuronal health, and reduced demyelination, respectively. Taken together, this data strongly supports the use of IV injection of AAV gene therapy as a safe and effective treatment for GM1 gangliosidosis.



289. CRISPR/Cas9-Mediated Therapeutic Editing of RPE65 Ameliorates the Disease Phenotypes in a Mouse Model of Leber Congenital Amaurosis

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ToolGen, Inc., Seoul, Korea, Republic of

Leber congenital amaurosis (LCA) is one of the leading causes of childhood-onset blindness without cure, characterized by autosomal recessive mutations in several genes including RPE65. In the study, we performed CRISPR/Cas9-mediated therapeutic correction of non-sense mutation on Rpe65 in rd12 mice which bears the corresponding human mutation. Subretinal injection of adeno-associated virus-delivered CRISPR/Cas9 and donor DNA, which resulted in >1% homology-directed repair (HDR) and ~1.6% deletion of pathogenic stop codon on Rpe65 in retinal pigment epithelial tissues of rd12 mice. Our approach effectively recovered scotopic a- and b-waves up to 21.2 ± 4.1% and 39.8 ± 3.2% of their normal counterparts in LCA mice at 6weeks after injection and thereafter up to 7 months. There was no definite evidence of histologic perturbation or tumorigenesis for 7 months during observation. Collectively, we present the first therapeutic correction of non-sense mutation on Rpe65 by HDR using CRISPR/Cas9, providing a new insight to develop therapeutics for LCA.

290. Safety of an AAVrh-Mediated APOE2 Treatment of Alzheimer's Disease in a Large Animal Model

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Alzheimer's disease (AD), a currently untreatable progressive degenerative central nervous system (CNS) disorder, affects 5.5 million Americans and is increasing in prevalence. There is no FDA-approved treatment for AD and despite nearly 1500 clinical trials across the world, there is no drug demonstrating long term efficacy. A major genetic risk factor for late onset AD are inherited variants of apolipoprotein E (APOE) alleles (E2, E3, and E4). APOE4 homozygotes have a markedly increased risk of developing AD, whereas, APOE2 homozygotes are protected from late onset AD. We hypothesized that genetic modification of the CNS of E4 homozygotes to express the protective E2 allele would prevent progressive neurologic damage. We have shown that expression of the protective APOE2 allele via gene transfer vectors was efficacious in reversing the effects of amyloid Aβ plaques in AD-mice [Zhao L, et al, *Neurobiology of Aging* (2016)], which lead to a study demonstrating that intracisternal or intraventricular administration of the AAVrh.10hAPOE2-HA vector in the CNS of nonhuman primates (NHP) resulted in persistent widespread expression of APOE2 across the CNS. To translate APOE2 gene therapy for AD to the clinic, we evaluated the safety of AAVrh.10hAPOE2-HA, an AAVrh.10 serotype coding for an HA-tagged human APOE2 cDNA delivered to the cerebral spinal fluid (CSF) of NHP (n=3). As a "worst-case" scenario, and to minimize the use of NHP, the animals were administered the vector [10^{14} genome copies (gc) total] via a combined intracisternal (cisternae magna; $5x10^{13}$ gc) and intraventricular (frontal ventricle; $5x10^{13}$ gc) routes. At multiple time points during the 8 wk trial, post-vector administration, NHP health was quantitatively evaluated for safety parameters, including hematologic, serum chemistry, blinded videotape analysis of behavior, magnetic resonance imaging (MRI) parameters, and pathology. The dual route vector-administered groups did not differ from the sham controls in any parameter. To assess for any vector- or surgery-related inflammation or acute pathology, the NHPs were evaluated by CNS MRI at 2 time points, prior to surgery (0 wk) and prior to necropsy (8 wk). Neither inflammation nor pathologic abscesses were observed. No pathology was noted beyond focal superficial parenchymal loss due to surgical intervention with the CNS catheters. The delivery to the CSF in the NHP brains appears to be well tolerated, suggesting that CNS delivery of AAVrh.10 coding for human APOE2 is safe. Combined with our earlier human clinical safety profile of AAVrh.10, this study supports the initiation of clinical trials with AAVrh.10hAPOE2 as a viable long-term treatment of homozygous AD patients.

291. Assessment of Bilateral Retinal Gene Therapy of the Phase 3 LCA2 Clinical Trial: A Longitudinal fMRI Study of Binocular Visual Functions

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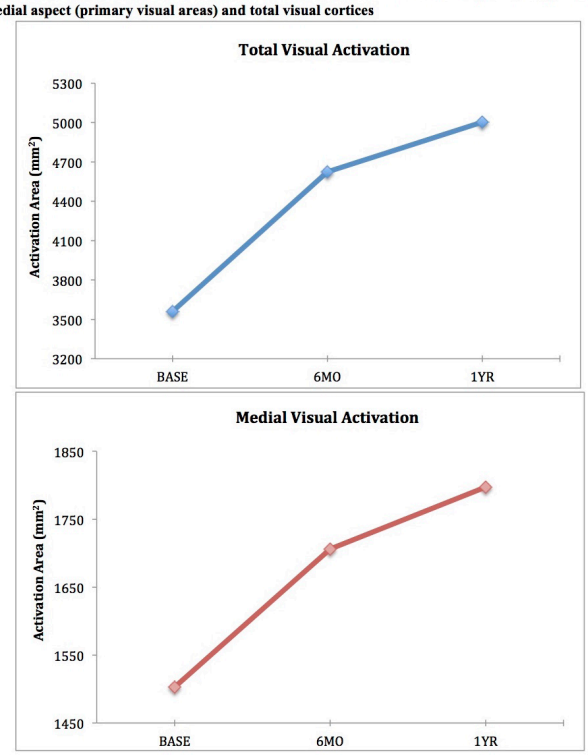
Introduction: Leber Congenital Amaurosis (LCA) is an inherited retinal dystrophy that is associated with mutations amongst 18 different genes[1]. The slower LCA disease progression associated with the *RPE65* mutation (LCA2) has made LCA2 patients the best candidates to undergo retinal gene therapy (GT). Cumulative positive results from all three LCA2 clinical trials, resulted in the recent FDA approval of the drug "*Luxturna*" for treatment of LCA2 disease. Similar to previous trials, the results for Phase 3 clinical trial also showed increased visual functions as a result of retinal GT. Here, we employed functional MRI (fMRI) as an objective method to independently explore the efficacy of GT on binocular visual functions of LCA2 patients. **Methods:** 9 LCA2 patients participated in this neuroimaging study. All subjects underwent MRI using a 3T equipped with a 12-channel head coil. Functional and structural MRI were performed at baseline (before intervention), and at 6 months and 1 year post bilateral administration of subretinal gene therapy separated by 10 days. For functional MRI (fMRI) participants binocularly viewed full-field checkerboard stimuli of varying contrasts (high, medium, and low) using MRI compatible video goggles to deliver visual stimuli. Structural scans were used to register participants' brains using the cortex based alignment (CBA) as implemented in BrainVoyager 20. Group averaged fMRI was performed using the multi-subject GLM analysis of BV-20 using a strict $p(Bonf) < 0.00005$ for the left hemisphere and $p(Bonf) < 0.00001$ for the right hemisphere. Based on previous report[2], cortical activations were quantified for areas of activations distributed over the total visual cortex and medial aspect (primary visual cortex). Final cortical activations were integrated over both the left and right cortices. **Results:** As shown in Figure 1.0 group averaged brain responses increase from baseline to one year after LCA2 patients received bilateral retinal gene therapy. Rate of increase for the areas of activations distributed in the left and right hemispheres were 40% and 20% for total visual cortex and the medial aspects, respectively.

Figure 1.0 Trajectories of cortical activation changes due to bilateral retinal gene therapy across the medial aspect (primary visual areas) and total visual cortices



Conclusions: Preliminary group fMRI results show the efficacy of *"Luxturna"* to reinstate vision in a group of LCA2 patients. LCA2 patients overall showed lower levels of cortical activations at baseline and after receiving retinal gene therapy, they presented significant increased levels of cortical activation at six months and one year. Results from the current study are consistent with recently reported results on Phase 3 clinical trial of LCA2 patients evaluated for the visual functions of both eyes[1]. Consistent to our previous report[2] fMRI results are indicative of the fact that the visual pathway in LCA2 patients remain responsive to visual stimulation once the remaining viable photoreceptors are restored through GT. This study demonstrates the role fMRI plays in providing complementary information to patients' clinical evaluation, and the potential it offers as an objective outcome measure for future retinal intervention studies.

References:

1-Russell, S., et al. (2017), *The Lancet*, vol 390 (10097) 849-860.
2-Ashtari, et al, (2017). *Ophthalmology,* 124(6), 873-883.

292. Route of Administration and AAV Serotype Significantly Impact Vector Distribution and Tropism in Mouse CNS

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Recombinant adeno-associated virus (AAV2) is the most common vector for clinical gene therapy of the CNS due to safety profile and longevity of expression in neurons. However clinical efficacy for neurologic disorders has been has been largely hindered because of relatively limited spread and transduction efficacy in large regions of the brain. In this study we compared the distribution, efficacy of transduction and cellular tropism of three commonly used AAV capsids (AAV9, AAV1, AAV2) expressing green fluorescent protein (GFP) as well as a modified AAV2 capsid that is unable to bind to heparan-sulfate proteoglycans (HSPG) (AAV2 HBKO), in mice following multiple routes of delivery including: intracerebroventricular (ICV), intra cisternal magna (ICM), and intraparenchymal (striatal and thalamic injections). Adult male mice were injected and brain and spinal cord were systematically analyzed for GFP expression using immunohistochemistry at 4 weeks post-injection. We found that the route of administration significantly altered the GFP expression pattern of all AAV vectors evaluated. ICV, ICM and intrathalamic delivery of AAV9-eGFP vectors led to robust GFP transduction in the brain. Intrathalamic and ICV administration of AAV9 transduced a majority of rostral-caudal brain structures, including cerebral cortex, striatum, hippocampus, thalamus, cerebellum and brainstem, while ICM injection mostly supported distribution of vectors in the midbrain and hindbrain regions, such as cerebellum and brainstem. AAV1 and AAV9 both demonstrated similar widespread transduction following thalamic injection; however AAV1 showed relatively limited distribution following ICV and ICM delivery compared to that of AAV9. Additionally, when compared to traditional AAV2, the modified AAV2 capsid (AAV2 HBKO) showed a more extensive distribution pattern following all the routes of administration evaluated. Unlike the parental AAV2 vector for which distribution is largely restricted to the injection site, AAV2 HBKO vector was capable of robust global brain distribution following a single injection into the thalamus or striatum indicative of bidirectional axonal transport. The vector serotypes evaluated also showed distinct profiles of cellular tropism in mouse CNS. While AAV2-HBKO transduced predominantly neurons, AAV1 and AAV9 transduced both neurons and astroglia in the brain. Furthermore, only AAV9 vectors resulted in widespread transduction of spinal cord motor neurons across all levels of the spinal cord when delivered either by ICV or ICM. Thus, AAV9 remains one of the preferred serotypes to target spinal cord motor neurons. These findings illustrate the importance of identifying the appropriate vector serotype in conjunction with the optimal route of delivery to selectively deliver transgenes of interest to different brain and spinal cord regions for the treatment of various CNS disorders.

293. Cas9/gRNAs Selective Targeting of the Beethoven *Tmc-1* Mutant Allele for Treating Progressive Hearing Loss by AAV-Based Delivery

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Hearing Loss (HL) is the most prevalent sensorineural disorder in humans affecting approximately 1/500 newborns and 4% of people at 45 years of age and younger, reaching 50% by 80 years of age. It is estimated that 360 million people worldwide have HL. Current treatment options focus on hearing aids and cochlear implants to bypass the sensory

deficit by amplifying sounds (hearing aids) or by encoding them as electrical impulses that are transmitted to the auditory nerve through an implanted electrode array (cochlear implant). Although these two habilitation options are effective, they do not restore "normal" hearing. Among alternative therapeutic strategies, gene therapy holds promise for 50% of pre-lingual deafness cases with a genetic cause. The transmembrane channel like 1 (Tmc1) was identified as the gene underlying both dominant and recessive forms of non-syndromic sensorineural HL at the DFNA36 and DFNB7/11 loci, respectively. DFNA36 hearing loss is post-lingual and progressive. One TMC1 mutation c.1253T>A (p.M418K) is orthologous to the murine Beethoven (Bth) mutation (Tmc1 c.1235T>A [p.M412K]). The natural progression of this hearing loss in humans is closely replicated in the Bth-heterozygous mouse ($Tmc1^{Bth/+}$). The $Tmc1^{Bth/+}$ mice initially have a slight hearing loss that rapidly progresses to profound deafness, while $Tmc1^{Bth/Bth}$ mice are profoundly deaf at the age of 3 weeks, when fully mature hearing can first be measured. We are developing an AAV-based gene editing strategy to prevent hearing loss using CRISPR technology to disrupt and knock-out the *Bth-Tmc1* allele and evaluate this strategy as a potential gene therapy to prevent hearing loss in humans. For this purpose we have designed 6 different gRNAs sequences that contain the *Bth* mutation at different positions. The sequences were evaluated in Mouse Embryonic Fibroblast-like cells from Beethoven mice constitutively expressing SpCas9. Cells transfected with 6 different gRNA-GFP plasmids were analyzed and allele disruption was determined by Sanger sequencing. One gRNA sequence was highly efficient in disrupting the *Bth* allele without targeting the *Wt* allele. To evaluate the system in a human cellular context, we designed 6 sequences targeting the human *Bth* allele and we evaluate their efficiency and specificity in human Haploid cells. Allele disruption was determined by Sanger sequencing and T7 assay. One gRNA sequence was highly efficient in disrupting the *Bth* allele without targeting the *Wt* sequence. To deliver the system into the mouse cochlea, AAV vectors have been identified that efficiently transduce cochlear outer hair cells. The AAV vector carrying the CRISPR system targeting the *Bth-Tmc1* allele will be delivered by intra-cochlear injection in the inner ear of $Tmc1^{Bth/+}$ mice to evaluate allele disruption and auditory function in-vivo.

294. Development of *Ex Vivo* Hematopoietic Stem and Progenitor Cell Gene Therapy for Friedreich's Ataxia

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Friedreich's ataxia (FRDA) is a multi-systemic incurable autosomal recessive neurodegenerative disease caused by a GAA repeat expansion mutation within the first intron of the frataxin (*FXN*) gene leading to a decrease of its expression. FRDA is characterized by ataxia, neurodegeneration, muscle weakness, and cardiomyopathy. Frataxin is a mitochondrial protein involved in iron metabolism. We recently reported the therapeutic efficacy of transplanting wild-type mouse hematopoietic stem and progenitor cells (HSPCs) into the YG8R mouse model of FRDA. Neurologic, muscular and cardiac complications were fully rescued in the HSPC-treated mice. Furthermore, degeneration of large sensory neurons was prevented in the dorsal root ganglia (DRGs) and mitochondrial function was improved in brain, skeletal muscle, and heart. Abundant HSPCs engrafted into affected tissues and differentiated into microglia in brain and spinal cord, and macrophages in DRG, heart and muscle, and led to frataxin transfer to the diseased neurons and myocytes. Given the high risks of morbidity and mortality associated with allogeneic HSPC transplantation, we are now developing an autologous HSPC gene therapy approach for FRDA. We will present our data and progress for an optimal *ex vivo* HSPC gene-correction strategy for FRDA.

295. Abstract Withdrawn

296. Engineering Micro-Indels in Glia Maturation Factor: a Novel Alzheimer's Disease Therapeutic Target

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Alzheimer's disease (AD) is a devastating, progressive neurodegenerative disorder that causes an irreversible cognitive decline in elderly patients. Currently, there is an urgent need for the development of an effective AD therapy. Our ultimate goal is to develop a robust AD patient-specific personalized, precision-guided, targeted gene editing and stem cell therapy. Neuroinflammation plays a significant role in the onset and progression of AD. Inflammatory response driven by the activation of the NLRP3 inflammasome in the microglia and secretion of proinflammatory cytokines contribute to β-amyloid (Aβ) aggregation and tau-mediated neurodegeneration that play a significant role in the onset and progression of AD. Glia maturation factor (GMF), a brain specific proinflammatory molecule discovered in our laboratory plays a crucial role in AD pathophysiology. We hypothesized that CRISPR-Cas9-mediated GMF gene editing in microglia is a novel approach to reduce neuroinflammation, neurodegeneration, AD pathophysiology and improve cognitive function. We developed a recombinant adeno-associated viral vector (AAV) and multiple lentiviral vectors (LVs) to engineer microinsertions and microdeletions in the GMF gene in BV2 microglial cells using CRISPR-Cas9 and GMF-specific sgRNAs. Confocal microscopy of BV2 cells transduced with an AAV simultaneously co-expressing nuclear localized *Staphylococcus aureus* CRISPR-Cas9 (SaCas9) and a GMF specific sgRNA revealed a small subset of BV2 cells expressing SaCas9 while completely lacking the expression of GMF, thereby confirming successful biallelic GMF gene editing. To further improve our GMF gene editing results, we generated a panel of LVs coexpressing either 1) *Streptococcus pyogenes* CRISPR-Cas9 (SpCas9), eGFP and Neo^r or 2) GMF-specific individual sgRNAs1-3, mCherry and $Puro^r$. BV2 cells were sequentially transduced with LV-SpCas9 and LV-GMF-sgRNAs1-3 to generate stable clones. Confocal microscopy revealed reduced GMF expression in the GMF-edited BV2 cells as compared to non-edited BV2 cells. DNA

sequencing of GMF edited clones revealed the presence of micro-indels in the exons 2 and 3 of the GMF coding sequence thereby conclusively proving SpCas9-mediated GMF editing in BV2 cells. Treatment of the wild type non-edited BV2 and GMF-edited BV2 cells with LPS revealed differences in basal p38 MAPK as well as LPS-induced phosphorylation of p38 MAPK. In wild type non-edited BV2 cells LPS treatment induced significant upregulation of pp38 MAPK, however in GMF-edited BV2 cells the pp38 MAPK levels were significantly lower, thereby indicating that GMF gene editing leads to attenuation of microglial activation. Overall, our data reveal successful AAV- and LV-mediated GMF gene editing in BV2 microglial cells. Our future *in vivo* studies will investigate the therapeutic potential of GMF gene editing in 5xFAD mouse model of AD. Our unique gene editing approach targeting neuroinflammation has a significant translational and therapeutic potential and will enable us to develop the next generation, precision guided and microglia-targeted gene editing AD therapy.

297. Delivery of an Artificial Transcription Factor for Angelman Syndrome Using AAV-PHP.B, AAV-PHP.eB and AAV-DJ/8J with Focused Ultrasound

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Angelman Syndrome (AS) is a rare neurogenetic disorder that arises from the genetic loss of the maternal *UBE3A* gene in the brain neurons, causing severe mental and physical impairments. The paternal allele has an intact *UBE3A* allele, but due to a brain-specific long non-coding RNA transcript, known as the UBE3A-antisense (*UBE3A-ATS*), paternal *UBE3A* remains silenced. Reactivation of the paternal allele could therefore restore UBE3A expression in the brain. One of the strategies for achieving reactivation is to repress the *UBE3A-ATS*. Previously it was shown that a zinc finger-based artificial transcription factor (ATF) called S1K, targeting the promoter of the *Ube3a-ATS* transcript, was able to repress the antisense strand and reactivate *Ube3a* transiently in the brain of a mouse (Bailus et al., 2016). Here we evaluate a strategy for using S1K to achieve long-term *UBE3A* expression in the brain. Recently described AAV vectors with improved capsids for enhanced neuronal transduction, such as AAV-PHP.B and AAV-PHP.eB and AAV-DJ/8J were tested for safety and distribution in *Ube3a*-YFP and C57BL/6J animals. Direct therapeutic injections to the brain were compared to systemic delivery. Since the AS brain requires a widespread *UBE3A* activation, one approach is the safe disruption of the blood brain barrier with focused ultrasound combined with microbubbles. Preliminary data will be presented on the safety and distribution of AAV containing S1K with different capsids (PHP.B, PHP.eB, DJ/8J), and the combination of focused ultrasound with microbubbles for wider distribution. After evaluation of the different delivery forms and routes of the proposed epigenetic therapy, the most promising approach will be tested for the duration of *Ube3a* activation and behavioral rescue in a mouse model of AS.

298. Gene Therapy for SUDEP and Autism Like Behaviors in Dravet Syndrome

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The aim of our laboratory is the development of therapeutic biological reagents for treating autism-like behaviors and sudden unexpected death in epilepsy (SUDEP) in neurodevelopmental disorders. Dravet Syndrome is a genetic disorder with abnormal brain activity during the postnatal developmental period. The incidence of Dravet Syndrome is approximately 1:16000. The average lifespan is 8 years old, and about 20% of Dravet Syndrome patients perish by SUDEP. More than 80% of patients with Dravet Syndrome have mutations in the SCN1A gene, encoding the sodium channel alpha subunit 1 (Nav1.1). In addition to SUDEP, patients with Dravet Syndrome display several types of severe seizures including generalized tonic-clonic seizures and febrile seizures. *Scn1a* heterozygous (*Scn1a*+/-) mice, a Dravet Syndrome mouse model with reduced Nav1.1 expression show (1) suppression of GABAergic inhibitory neuronal activity, (2) generalized tonic-clonic seizures, febrile seizures and SUDEP around the 4th postnatal week, and (3) autism-like behaviors in adulthood. Dravet Syndrome patients are typically resistant to anti-epileptic drugs, and there are currently no long-lasting and effective treatments for the syndrome. We hypothesize that the endophenotypes of Dravet Syndrome in *Scn1a*+/- mice will be ameliorated by long-lasting activation of inhibitory GABAergic neurons in the brain. Here, we report on the properties of an AAV vector encoding a sodium channel subunit driven by a GABAergic promoter to prevent SUDEP, seizures, and autism-like behaviors in *Scn1a*+/- mice. This work was supported by the Canadian Institutes for Health Research, Dravet Canada, and the Dravet Foundation USA.

299. Sequence Integrity of Codon-Optimized RPGR Construct Maintained *In Vitro* and *In Vivo*

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Purpose: AGTC is developing an AAV-based gene therapy product (AAV2tYF-GRK1-*hRPGRco*) to treat X-linked retinitis pigmentosa (XLRP) caused by mutations in the retinitis pigmentosa GTPase regulator (*RPGR*) gene. The retinal ORF15 isoform of *RPGR* contains a highly repetitive purine-rich region making the natural form unstable and prone to mutations during cloning and vector production. We successfully designed and synthesized a codon-optimized human cDNA (*hRPGRco*) that was shown to be stable through multiple passages in plasmids, in recombinant herpes simplex viruses and during AAV vector production. Here, we further demonstrate the construct integrity of the *hRPGRco* gene in *in vitro* and *in vivo* models treated with our drug product. In addition, we also examined the size, integrity, glutamylation and functionally crucial protein interactions of vector-expressed RPGR protein in HEK293 cells and retinal tissues

of RPGR deficient Rd9 mice treated with rAAV2tYF-GRK1-*hRPGRco*. **Methods:** DNA/RNA sequencing of the *hRPGRco* was based on the overlapping DNA fragments obtained by PCR or reverse-transcription-PCR (RT-PCR) from total DNA extracted from retinal tissues of RPGR mutant (XLPRA2) dogs or RNA extracted from retinal tissues of Rd9 mice subretinally injected with AAV2tYF-GRK1-*hRPGRco*. For DNA sequencing, treated XLPRA2 dogs were sacrificed 6-8 weeks post-injection. Retinal tissues were collected, snap frozen and then processed for DNA extraction. The resulting DNA was used as template for PCR amplification of the *hRPGRco* transgene from the AAV vector-transduced photoreceptor cells. For RNA sequencing of *hRPGRco* transcripts in vector-treated Rd9 mice, treated animals were sacrificed at 6 weeks post-injection. Retinal tissues from vector treated eyes or untreated control eyes were processed for RNA extraction. The resulting total RNA was used as a template for RT-PCR amplification of the *hRPGRco* mRNA transcribed from the injected vector. Vector-expressed RPGR protein in transduced HEK293 and Rd9 mice retinal tissues was examined by western blot and liquid chromatography mass spectrometry (LC-MS/MS). In addition, posttranslational modification (glutamylation) and binding ability to RPGR regulator-interacting protein 1 (RPGRIP1) were also evaluated. **Results:** The assembled Sanger read contigs for DNA and cDNA (derived from mRNA) isolated from rAAV2tYF-GRK1-*hRPGRco* treated retinal tissues were 100% identical to the reference *hRPGRco* sequence. Western blot revealed stable RPGR protein expression in HEK293 cells and Rd9 mice. The spacing of proteolytic sites in the highly repetitive glutamic acid rich region of ORF15 prevented the cleavage to detectable oligopeptides by LC-MS/MS analysis and led to the coverage of 76.6% of all amino acids in the protein sequence which was confirmed to be identical to *hRPGRco* reference sequence. Vector-expressed RPGR was shown to be glutamylated (GT335-immunoreactive) and able to bind to RPGRIP1, an interaction that is required for its function in connecting cilium of photoreceptors. **Conclusion:** Codon-optimized *hRPGR* cDNA in the rAAV2tYF-GRK1-*hRPGRco* vector maintained its integrity during transduction and transcription *in vitro* and *in vivo*. Vector-expressed RPGR protein was glutamylated and able to bind to its partner RPGRIP1 to fulfill its functional role in retinal cilium.

300. Programmable Transcription of CDKL5 in Precision Disease Models of CDKL5 Deficiency Disorder

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Neurological diseases are a heterogeneous group of disorders caused by alterations in nervous system function. Due to technological advances over the last decade; many of these disorders can be attributed to genetic factors such as small or large structural aberrations or point mutations. Early onset epileptic encephalopathies are among the most devastating conditions in pediatric populations. CDKL5 deficiency disorder (CDD) is a severe neurodevelopmental X-linked dominant disorder caused by mutations in cyclin-dependent kinase like 5 (CDKL5). Females with CDD undergo X-chromosome inactivation forming a mosaic of neurons expressing the mutant or wild type allele of CDKL5. Our research is focused on methods to reactivate the wild type allele of CDKL5 on the silenced X-chromosome in CDD patient cells and cell lines. Here, we modeled pathogenic single nucleotide variants in the catalytic domain of CDKL5 that are causative for CDD using CRISPR/Cas9-mediated homology directed repair and novel Cas9-based adenine base editors (ABE) in immortalized neuronal cells. Further, we have identified and validated several cis regulatory elements that can regulate CDKL5 expression in human CDD cells. This technique utilizes CRISPR guide RNAs (gRNAs) with deactivated Cas9 fused epigenetic effector domains targeted to the promoter and putative enhancer regions of the CDKL5 gene and result in significant upregulation of the gene when transfected with a pooled group of gRNAs. However, our current strategy does not allow for allele specific control of CDKL5 activation. Future studies will utilize combinatorial approaches using pharmacological approaches in conjunction with our established programmable transcription. We believe this to be the first description of programmable transcription in a disease model of CDD. This approach holds great potential for children suffering from CDD.

301. The Green Monkey as a Test Subject in AAV Research

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Nonhuman primates (NHP) play a pivotal translational role in preclinical gene therapy studies evaluating new therapeutic vectors. With genetic, physiologic, anatomic and immunological homology closely shared with humans, old world NHPs allow critical preclinical data to be generated related to achievable delivery, biodistribution, transgene expression, timecourse of expression and tolerance. The St. Kitts green monkey (*Chlorocebus sabaeus*) has proven particularly suited to *in vivo* assessment of AAV vectors as they are free of many of the zoonoses and reverse zoonoses common to continental NHP populations and display low seroprevalence to a variety of AAV serotypes due to lack of pre-exposure and subsequent immunological response. Serology results from our NHP research facility over several decades have continued to confirm low or absent seropositivity to a variety of AAV capsid variants confirming the St. Kitts green monkey as an ideal NHP test system to perform IND-enabling large animal biodistribution, pharmacokinetic, pharmacodynamic, safety/toxicity, and efficacy studies. We performed a meta-analysis of both published studies and anonymized experimental data and methods performed at RxGen's testing facility involving AAV vector delivery in the green monkey. The culmination of these studies highlights the green monkey's therapeutic value across multiple therapeutic disease spaces from a diverse range of study types utilizing multiple dosing delivery routes.

302. Improving Informed Consent for High Risk Early Phase Clinical Trials: a Systematic Review of Tested Interventions

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Introduction: A "standard" informed consent procedure might not be optimal for clinical trials that involve complex scientific underpinnings and significant uncertainty about risks, such as early phase clinical trials of cell- and gene-based therapies. We previously examined barriers to obtaining a truly informed consent, including enhancing understanding and limiting the therapeutic misconception, with a focus upon Parkinson's disease (PD). This process is particularly challenging in neurodegenerative conditions due to their incurable nature, cognitive dysfunction, and progressive decline. With recent, ongoing, and planned early phase clinical trials in various neurodegenerative conditions, including PD, we therefore examined studies of tested interventions that might enhance the informed consent process, including the use of new technologies such as multi-media interventions. **Methods:** We performed a systematic review of the literature published since 2011 using various combinations of search terms with *primary subject headings* 'informed consent', 'consent' multiple modifiers, and *outcome terms* 'comprehension', 'readability', 'pre-post testing', 'decision making', 'patient', 'satisfaction' in *databases including* PubMed, Cochrane Library, EMBASE. Studies were eligible for inclusion if (1) they assessed informed consent effectiveness, or (2) described novel informed consent tools and assessed measurable effects on participants. **Results:** Of 4200 articles returned from the search, a subset of 900 were accepted for further review. Of these, 16 were identified that met criteria for further evaluation. In these publications, participants included adult and pediatric populations and, although various fields were included, studies of informed consent in cancer clinical trials were dominant. Only one focused on a psychiatric disorder (schizophrenia). Eleven studies assessed the use of technology, e.g., tablets, videos, multimedia, in the consenting process. Others assessed non-technological means, including targeted education sessions based upon prior administration of questionnaires. Most of the studies demonstrated effects of their respective interventions, including higher rates of comprehension of study procedures, higher rates of deciding upon study participation, and improved understanding and retention of information presented in the consent document. In one study, children but not adults benefited more than controls from information presented on a tablet rather than traditional paper format. **Conclusions:** Effectively communicating complex information to potential subjects, study partners, and family members about the importance of clinical trials and their risks and benefits can be challenging. Targeting our consenting methods to provide more accessible information, using interactive means of judging understanding, and employing various technologies to better visually conceptualize key points may improve informed consent for challenging neurodegenerative studies. It may also increase enrollment as subjects understand the importance of these studies. However, little information exists to determine how well such interventions will perform in early phase clinical trials with complex scientific underpinnings, such as gene and cell therapy clinical trials. This gap in knowledge needs to be addressed as such trials are increasing in number.

574. Modeling Chronic Gut Motility Deficiency in Autistic Children Using Patient-Derived Induced Pluripotent Stem Cells

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Many children with Autism Spectrum Disorder (ASD) suffer from decreased gut motility that manifests as debilitating chronic constipation, leading to an unhealthy aversion to food. We hypothesized that the same neurological defects that cause the hallmark cognitive impairments in ASD also negatively impact the enteric nervous system controlling gut peristalsis. Within the autism spectrum, we chose to study one particular condition called Phelan-McDermid Syndrome (PMDS), because *every* patient with the diagnostic chromosome 22 micro-deletion has a dramatic decrease in gut motility. Evidence suggests that the gene-of-interest in the deleted region is SHANK3 because mice with SHANK3 haploinsufficiency show decreased postsynaptic density of neurotransmitter receptors. The overriding goal of this project is to develop novel drug treatments for patients with PMDS and ASD. Finding new compounds that can increase (or bypass) postsynaptic receptor density could alleviate these patients' gut motility problems. However, models to study the enteric nervous system either involve invasive biopsy procedures to remove enteric tissue from patients or the use of animal models that fail to replicate the pathology observed in humans. Therefore, the objective of this study is to create a human disease-in-a-dish model of enteric nervous system dysfunction by generating stem cells from PMDS patients and differentiating them into enteric neurons (ENs). We predict that molecular characterization of SHANK3-deleted enteric neurons will identify aberrant signaling pathways suitable for therapeutic intervention. Preliminary experiments from our laboratory have demonstrated the feasibility of differentiating patient-derived induced pluripotent stem cells (iPSCs) into neural crest (NC) cells, which are necessary precursors to making enteric neurons. Figure 1 shows normal iPSCs differentiated to NC cells through the activation of Wnt and inhibition of SMAD signaling pathways over 17 days in culture. Immuncytochemistry revealed that NC cells stained positive for P75 and AP2, but did not express PAX6, a marker for central nervous system development. NC plus smooth muscle co-culture experiments are currently underway to drive fluorescently labeled normal and SHANK3-deficient NC cells to enteric neurons, thus enabling phenotypic characterization of the disease phenotype *in vitro.*

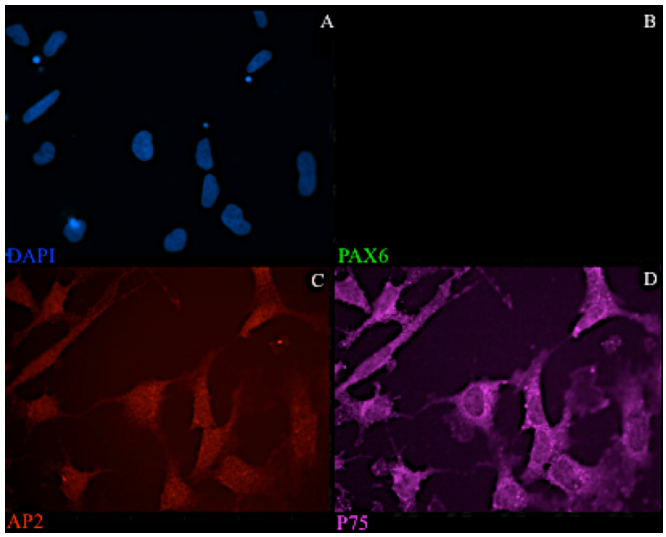

Figure 1: Immunocytochemistry of IPSC-derived neural crest cells A) DAPI. B) Negative expression for PAX6, an essential protein in central nervous system neuron development but absent in peripheral nervous system development. C) Positive expression for AP2, a marker expressed in neural crest cells. D) Positive expression for the nerve growth factor receptor P75, which is expressed in central and peripheral nervous system lineages.

303. Transduction of Photoreceptor and Pigmented Epithelial Cells Following a Single Subretinal Injection of AAVHSC17 in Minipigs

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A novel group of Clade F adeno-associated viruses have been isolated from normal human CD34+ hematopoietic stem cells (AAVHSCs) and have shown high-efficiency nuclease-free gene editing as well as gene transfer capabilities. In non-human primate biodistribution studies, we have observed high-level transduction of retinal cells following a single intravenous delivery of AAVHSC demonstrating that AAVHSCs have tropism for the mammalian retina. The ability of AAVHSCs to also transduce retinal cells following localized delivery to the eye was evaluated. AAVHSC17 packaging a chicken beta actin-promotered self-complementary GFP transgene (AAVHSC17-CBA-scGFP) was prepared by triple transfection in HEK293 cells and purified through two rounds of CsCl density gradient ultracentrifugation. Anesthetized Göttingen minipigs received a single subretinal injection (0.1 mL/eye) in both eyes of either formulation buffer control (n=1) or AAVHSC17-CBA-scGFP, 1.3 x 10^{12} vg (n=2). Eyes were examined by slit-lamp biomicroscopy and/or indirect ophthalmoscopy following completion of each treatment to confirm location and appearance of the dose. Body weights and food consumption were monitored weekly and twice daily, respectively. Ophthalmic examinations were performed pre-study and on days 3, 8, 15, and at sacrifice on day 28 post-dosing. Spectral domain optical coherence tomography (SD-OCT) for GFP autofluorescence was performed once pre-study and on day 28. At sacrifice, animals were perfused with saline followed by 4% paraformaldehyde, tissues were collected, placed in 4% paraformaldehyde for 24 h, embedded in OCT and frozen. Sections were prepared and analyzed for direct GFP fluorescence and GFP expression by immunohistochemistry (IHC). Animals remained in good health throughout the study. Neither treatment-related ophthalmic changes nor ocular inflammation were observed. SD-OCT images showed regions of GFP autofluorescence that corresponded to the dosing bleb and surrounding tissue in animals treated with AAVHSC17-CBA-scGFP. GFP expression, as determined by direct fluorescence and IHC, was observed in all retinae, optic nerves, optic chiasmata, and optic tracts from animals treated with AAVHSC17-CBA-scGFP. GFP expression was in all retinal layers with greater intensity in cells of retinal pigmented epithelium, photoreceptors, and outer nuclear layer. GFP expression in optic nerves, optic chiasmata and optic tracts was multifocal, less intense and in filament (axon)-shaped structures, while other brain regions examined were negative. No GFP expression was noted in ocular tissues and brains of animals treated with formulation buffer alone. Taken together, these data demonstrate that administration of AAVHSC17-CBA-scGFP by subretinal injection in minipigs was well-tolerated and resulted in GFP expression in all retinal layers. These studies suggest that AAVHSCs may be useful as therapeutic vectors for treating diseases of the eye in humans.

Oligonucleotide Therapeutics I

304. TANGO - Targeted Augmentation of Nuclear Gene Output - for the Treatment of Genetic Diseases

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Most human genetic diseases are due to loss or reduction of function of a single gene, and currently approved therapeutic strategies are unable to directly increase protein expression to treat most of these inherited deficiencies. Thus, there is a severe lack of curative therapies for most monogenic diseases, and patients and their physicians must rely on palliative care. Stoke Therapeutics is developing first-in-class medicines to treat such monogenic diseases by leveraging antisense oligonucleotides (ASOs), an established and FDA-approved therapeutic modality. Unlike other antisense approaches, Stoke's approach, TANGO (Targeted Augmentation of Nuclear Gene Output), exploits naturally-occurring non-productive splicing events to increase target protein expression via modulation of splicing. Importantly, TANGO does not correct aberrant splicing resulting from mutation and does not alter protein coding splicing isoforms. To determine the scope and scale of this novel target landscape, Stoke has built a proprietary bioinformatics pipeline that has identified tens of thousands non-productive splicing events amenable to the TANGO technology, and over 50% of these events are not annotated in RefSeq databases. Evaluation of these events has identified nearly 2,000 potentially druggable monogenic disease-associated genes. ASO screening of prioritized disease targets has yielded potent and gene-specific up-regulation for multiple targets. One of our programs is Dravet syndrome (DS), a severe epileptic encephalopathy characterized by high seizure frequency and severity, as well as cognitive impairment. The majority of DS patients carry *de novo* mutations in the *SCN1A* gene leading to haploinsufficiency of

the voltage-gated sodium channel alpha subunit (Na_V 1.1). We have identified several ASOs that significantly increase the expression of *SCN1A* in neural-progenitor cells, as well as differentiated neurons. We have determined that the increase in gene expression results from a gene-specific reduction of non-productive mRNA and an increase of productive mRNA. In addition, intracerebroventricular (ICV) injection of the lead ASO in neonate and adult mice yielded a significant and dose-dependent increase in SCN1A mRNA as well as Na_V 1.1 protein. Moreover, the lead ASO had no significant effect on other voltage-gated sodium channel family members. Timecourse experiments in both, neonate and adult mice showed a sustained increase in *SCN1A* expression from a single, bolus ICV injection of the lead ASO. These results indicate that Stoke's ASO technology could provide the first gene-specific, disease-modifying approach to restore physiological Na_V 1.1 levels to prevent seizures and reduce developmental deficits. Stoke's technology offers a pioneering strategy to treat any disease that results from reduced expression or insufficient activity of a gene that contains a non-productive splicing event, and since over 50% of genes possess such a signature, the potential target opportunity is significant and largely untapped.

305. Endogenous miRNA Competition as a Mechanism of Therapeutic shRNA-Induced Muscle Toxicity

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RNA interference (RNAi) is a promising gene therapy strategy for targeting dominant mutations. This therapy uses the delivery of small hairpin RNAs (shRNAs) to knock down gene expression; however, delivery of too much shRNA can interfere with the normal function of microRNAs (miRNAs) in targeted organs, leading to toxicity. We previously delivered recombinant adeno-associated viral (rAAV) vectors expressing shRNAs targeting the liver in mice, and discovered that the cause of liver toxicity was shRNA competition with miR-122-5p miRNAs, which consequently de-repress miR-122 target mRNAs. Tissues other than liver do not express miR-122, despite many reports of shRNA-induced toxicity. While improved shRNA and vector design strategies can now be incorporated avoid toxicity, the underlying consequences of excessive shRNA expression in other tissues is still unclear. We now seek to understand the effect that excessive shRNAs have on muscle miRNAs, using a high throughput small RNA sequencing strategy that simultaneously captures exongenous shRNAs and endogenous miRNAs. We delivered $2x10^{12}$ vector genomes of rAAV6 vectors expressing shRNAs to mice by tail vein injection. The shRNAs were driven by the U6 promoter and targeted beta-galactosidase mRNA (rAAV-U6-shRNA). We then performed small RNA sequencing on liver samples and several muscle samples, including the diaphragm, gastrocnemius, quadriceps, tibialis anterior, and heart. Sequencing was performed in biological triplicates of mice at two weeks or six weeks after administration of rAAV-U6-shRNA, and both 19- and 21-nucleotide (nt) complementary stem sequences were compared. shRNA expression was highest in the heart, reaching amounts exceeding the level of miRNAs by six weeks after transduction. Mice injected with the 19-nt shRNA showed no phenotypic changes, while those injected with the 21-nt shRNA exhibited substantial cardiomyopathy. miR-1 was overall the most abundant miRNA in the muscle tissues examined, and was reduced after injection of the 21-nt shRNA, as compared with the 19-nt shRNA - most notably in the diaphragm. Furthermore, we found a significant reduction ($p = 0.01$) in the most abundant isoform of miR-1 relative to other miR-1-3p isoforms in all evaluated muscle tissue samples. This pattern is similar to the shRNA competition we had previously observed with a 22-nt isoform of miR-122-5p in the liver. Our data suggest that certain miRNAs, as demonstrated here with miR-1, may compete with exogenously delivered shRNAs, leading to the observed cardiomyopathy. Understanding how shRNAs compete with tissue-specific miRNAs will allow us to develop safer and more effective therapeutic gene knockdown strategies.

306. Non-Native Conformational Isomers of Catalytic Domain of PCSK9 Induce an Immune Response, Reduce Lipids and Increase LDL Receptor Levels

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Non-native conformational isomers of PCSK9 induce immune response, reduce lipids and increase LDL receptor levels PCSK9 (Proprotein convertase subtilisin/kexin type 9) plays an important role in regulating plasma cholesterol levels by promoting hepatic LDL receptor degradation. The current developed monoclonal antibody inhibitors block the interaction between PCSK9 and LDL receptor, hence, significantly decrease plasma cholesterol levels and provides beneficial therapeutic effects on clinic outcome. To reduce the action of PCSK9 in plasma, we proposed a novel strategy to produce a panel of non-native conformational altered isomers of PCSK9 (X-PCSK9) to develop active immunotherapy targeting at native PCSK9 to inhibit/block the interaction of PCSK9 with LDL receptor to decrease plasma cholesterol levels. We used scrambled disulfide bond technique to generate conformational altered isomers of the catalytic domain (residues 152 to 452) of mouse PCSK9. We focused on four X-isomers, X-PCSK9-A1, A2, B1 and B2 and on their immune responses and effects on plasma cholesterol and triglyceride levels in both *C57BL/6J* and *Apoe-/-* mice. We showed that the four immunogens produced significant immunogenicity against native PCSK9 to day 120 after immunization of *C57BL/6J* and *Apoe-/-* mice. These four immunogens resulted in significantly decreased of plasma cholesterol levels in *C57BL/6J* mice, whereas only X-PCSK9-B1 decreased plasma cholesterol levels in *Apoe-/-* mice. X-PCSK9-B1 immunogen decreased plasma triglyceride levels in both *C57BL/6J* and *Apoe-/-* mice. The X-PCSK9-B1 treated mice had increased LDL receptor mRNA and protein levels at day 120 after treatment. Thus, this study provided a new potential promise of long-term immunotherapy for the treatment of hypercholesterolemia.

307. A Novel Strategy for Gene Therapy of Neuropathic Pain through siRNA-IRF5 with Homing Peptides to Microglia

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[Background] Neuropathic pain is difficult to be satisfactorily treated by current medication. It is urgent to be developed the novel therapy for the pain. Recently, the homing peptides targeting to specific tissues receive a great deal of attention for their potential of gene delivery. And, interferon regulatory factor 5 (IRF5) has been reported to be involved in the pathogenesis of neuropathic pain. IRF5 is mainly expressed in M1-type microglia and is upregulated by spinal nerve injury, which induces the expression of ATP receptors, such as the P2X4 receptor, to activate microglia and signal neuropathic pain in the spinal cord. Therefore, we hypothesized the complexes between the homing peptides and therapeutic oligonucleotides could be a novel strategy of treatment. [Objective] To identify the homing peptides to microglia and establish a technology to deliver small RNA to microglia by the peptides for treatment of neuropathic pain. [Material and methods] (1) To identify the homing peptides to microglia, three cycles of both *in vivo* and *in vitro* biopannings were performed by using C7C phage library in mice spinal cord and in M1- and M2-type microglia. (2) After screening of the homing peptides, the most frequent candidate peptides, MG1 for M1 microglia and MG2 for M2 microglia, were synthesized and were applied to cultured microglia cells and wild type mice to confirm the specificities of the peptides. (3) The peptides bound with IRF5-siRNA were applied to LPS-stimulated microglia to evaluate the knockdown effect. And the complexes were administrated into neuropathic pain model mouse through intrathecal lesion for the treatment of neuropathic pain. [Results] (1) Fifty five kinds of homing peptides were totally identified in M1 and M2 microglia. MG1 peptide was detected at 13 times in 51 sequences as the candidate homing peptide for M1 microglia, and MG2 peptide for M2 microglia was detected at 8 times in 50 sequences. (2) MG1 and MG2 peptides were specifically labeled with cultured M1 or M2 microglia compared with CD86 for M1 marker or CD206 for M2 marker. *In vivo* study, MG1 and MG2 were observed with Iba1 staining for microglia marker in spinal cord. MG1 were observed more than MG2 peptide in the sections of spinal cord. (3) MG1+IRF5-siRNA or MG2+IRF5-siRNA were effectively suppressed gene expression of IRF5 in microglia cells. In addition, MG1+IRF5-siRNA were successfully suppressed hyperalgesia with high efficiency in neuropathic pain model mice. [Conclusion] We provide a novel gene therapy for the treatment neuropathic pain, which has high potential to be applied to the patient. This strategy will realize the pinpoint delivery of siRNA and minimize the off-target effects.

308. Redox Sensitive Metastable DNA Junctions for Multivalent Delivery of Therapeutics

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Delivery is one of the most challenging technical barriers for effective use of oligonucleotide therapeutics (OT) *in vivo*. Recent studies have demonstrated that simple nucleic acid nanostructures can act as molecularly uniform, multivalent carrier platforms for therapeutic cargos and cell targeting ligands. Although this approach has significant promise, much remains unknown regarding the effects of carrier nanostructures on the (I) *in vivo* distribution and pharmacokinetics, (II) cell entry and endosomal escape and (III) cytoplasmic processing of therapeutic cargos such as small interfering RNAs (siRNA). To examine these issues and improve biological effects, we have developed a novel nucleic acid three-way junction (3wj) that can be engineered to overcome physiological barriers and respond to biochemical cues encountered at different stages of delivery. These junctions are self-assembled at low temperature (4°C) by Watson-Crick base-pairing and then covalently stabilized by crosslinkers that bridge proximal attachment sites on complementary strands. The crosslinkers reinforce the 3wjs against disassembly (at 37°C) and biochemical degradation during delivery, and allow the constructs to be unusually compact compared to normal nucleic acid junctions stabilized by Watson-Crick base-pairing alone. By using cleavable, reducible, or acid labile crosslinkers, the 3wjs can be engineered to dissociate at a specific stage of the delivery process. We demonstrate design, assembly and efficient crosslinking of compact 3wjs using disulfide based crosslinkers. We show that these junctions are highly stable in extracellular media but can readily dissociate in reducing environments such as the cytosol. Variants of these junctions carrying pairs of heterogeneous siRNAs allow highly efficient RNAi knockdown of combinatorial targets, and incur no penalty in RNAi activity when compared to individual siRNAs. Our results give a new class of simple nanostructure with advantageous properties for therapeutic delivery and useful functions for probing cellular uptake and processing pathways affecting OTs.

309. MicroRNA-219 Reduces Viral Load and Pathologic Changes in Theiler's Virus-Induced Demyelinating Disease

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Analysis of microRNA (miR) expression in the central nervous system white matter of SJL mice infected with the BeAn strain of Theiler's murine encephalomyelitis virus (TMEV) revealed a significant reduction of miR-219, a critical regulator of myelin assembly and repair. Restoration of miR-219 expression by intranasal administration of a synthetic miR-219 mimic before disease onset ameliorates clinical disease, reduces neurogliosis, and partially recovers motor and sensorimotor function by negatively regulating proinflammatory cytokines and virus RNA replication. Moreover, RNA-sequencing of host lesions showed that miR-219 significantly downregulated two genes essential for the biosynthetic cholesterol pathway, *Cyp51* (lanosterol 14-α-demethylase) and *Srebf1* (sterol regulatory element-binding protein-1), and reduced cholesterol biosynthesis in infected mice and rat CG-4 glial precursor cells in culture. The change in cholesterol biosynthesis had both anti-inflammatory and anti-viral effects. Because RNA viruses hijack endoplasmic reticulum double-layered membranes to provide a platform for RNA virus replication and are dependent on endogenous pools of cholesterol, miR-219 interference with cholesterol biosynthesis interfered virus RNA replication. These findings demonstrate that miR-219 inhibits TMEV-induced demyelinating disease through its anti-inflammatory and anti-viral properties.

310. Development of an Effective Platform to Deliver Protein Therapeutics in the Retina In Vivo

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Purpose: There is currently no efficient method to deliver proteins across the plasma membrane of photoreceptor or retinal pigment epithelium (RPE) cells in vivo. We have developed a platform protein delivery system that can transport proteins into retinal cells including ganglion cells, photoreceptors and retinal pigment epithelium via intravitreal injection. **Methods:** Nucleolin was used as a potential receptor to introduce proteins intracellularly in the retinal cells of mice. The aptamer AS1411 can bind to nucleolin and cross the plasma membrane of retina invivo. Biotinylated AS1411 complexed with Traptavidin was used as a platform to deliver proteins GFP (AS1411/Traptavidin/GFP complex)or X-linked inhibitor of apoptosis (XIAP; AS1411/Traptavidin/XIAP complex) by using intravitreal injection in BALB/C mice. Frozen sections were analyzed for uptake of proteins in the retina. Apoptosis was induced by injecting 20 nmol NMDA into the vitreous. NMDA/XIAP complex injected eyes were compared with NMDA/GFP complex injections to test XIAP function. Retinas were harvested at 24 hours and TUNEL analysis was used to assess apoptosis. Caspase assay was performed to detect the differences in Caspase 3 and 7 activity. **Results:** Intravitreal injection of GFP or XIAP complexes enabled delivery of the proteins to ganglion cells, photoreceptors,and retinal pigment epithelium in vivo. XIAP conferred significant protection to retinal cells in NMDA induced apoptosis when compared to GFP. NMDA/XIAP complex showed fewer apoptotic nuclei by TUNEL analysis relative to NMDA/GFP complex injected eyes. Caspase assay also confirmed a decrease in activity of Caspase 3/7 in XIAP treated eyes compared to GFP. **Conclusions:** AS1411/Traptavidin complex can be used as a platform to deliver therapeutic proteins to the retina. Rapid transport of XIAP protein into the cells and inhibition of apoptosis without significant toxicity was observed. This system can be potentially utilized to introduce a large variety of therapeutically relevant proteins that are previously well characterized to maintain the structural integrity and function of retina, thus, preventing vision loss due to ocular complications.

311. VSMC Specific Aptamer Ligands for the Next Generation of DES to Prevent Neointimal Formation

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Pathological vascular smooth muscle cell (VSMC) activation is responsible for many vascular disorders, including in-stent restenosis. Activated VSMCs dedifferentiate, migrate and proliferate resulting in neointimal formation. Strategies to prevent VSMC activation, such as cell growth inhibitors used in drug eluting stents (DES), have the undesired effect of impairing re-endothelialization. This attenuation of endothelial healing increases the risk of neoatherosclerosis, late stent thrombosis and death. Selectively inhibiting VSMC growth would greatly improve the safety and efficacy of DES. To identify agents that distinguish between VSMCs and endothelial cells (ECs), we generated nuclease-resistant VSMC-specific aptamer ligands. Aptamers are synthetic, structured oligonucleotide ligands that recognize targets with similar specificity and affinity as antibody-antigen interactions. We previously reported on a VSMC-specific aptamer that inhibits VSMC migration to multiple agonists, including platelet-derived growth factor-BB (PDGF-BB), and prevents neointimal formation in vivo following acute vascular injury (Thiel et. al. Molecular Therapy 2016). We have now identified two additional VSMC aptamers that modulate VSMC, but not EC, proliferation and apoptosis, respectively, through unknown mechanisms. More recently, we have developed a novel approach, which combines CRISPR technology with our innovative high-throughput Aptamer Fluorescence Binding and Internalization (AFBI) assay (Thiel et. al. Methods 2016), in order to enable the identification of the anti-proliferative aptamer and pro-apoptotic aptamer protein targets. These ongoing studies to identify the aptamer cell-surface targets will provide a basis of developing agents, including the aptamers themselves, that can distinguish VSMCs from ECs to improve the efficacy and safety of DES. The VSMC-specific aptamer ligands represent a timely convergence of clinical need, basic science discoveries of mechanisms of neointimal formation, and drug development to generate the next generation of therapies for vascular diseases.

312. Targeting EGFR & HER2 & HER3 with a Three-in-One Aptamer-siRNA Chimera Confers Superior Antitumor Activity in HER2 Expressing Breast Cancer

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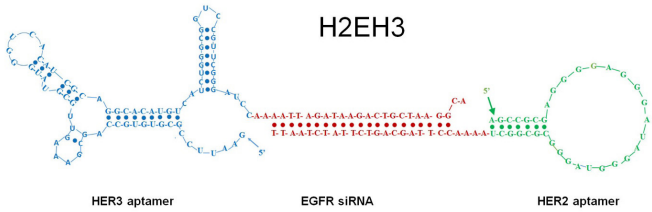
HER family members are interdependent and functionally compensatory. Simultaneously targeting EGFR&HER2&HER3 by antibody combinations has demonstrated the superior treatment efficacy over targeting one HER receptor. However, antibody combinations have their limitations in high immunogenicity and high cost. In this study, we have developed a three-in-one nucleic acid aptamer-siRNA chimera, which targets on EGFR&HER2&HER3 in one molecule. This inhibitory molecule was constructed such that a single EGFR siRNA is positioned between HER2 and HER3 aptamers to create a HER2 aptamer-EGFR siRNA-HER3 aptamer chimera (H2EH3). EGFR siRNA was delivered into HER2 expressing cells by HER2&HER3 aptamers-induced internalization. HER2&HER3 aptamers act as antagonist molecules for blocking HER2 and HER3 signaling pathways and also as tumor targeting agents for siRNA delivery. H2EH3 enables down-modulation of the expression of all three receptors, thereby triggering cell apoptosis. In breast cancer xenograft models, H2EH3 is able to bind to breast tumors with high specificity, and significantly inhibit tumor growth via either systemic or intratumoral administration. Owing to low immunogenicity, ease of production, and high thermostability, H2EH3 is a promising therapeutic to supplement current single HER inhibitors and may act as a treatment for HER2 positive breast cancer with intrinsic or acquired resistance to current drugs.



313. Splice-Switching in Tumor Cells by Artificial Cationic Lipopeptide-PMO Conjugates

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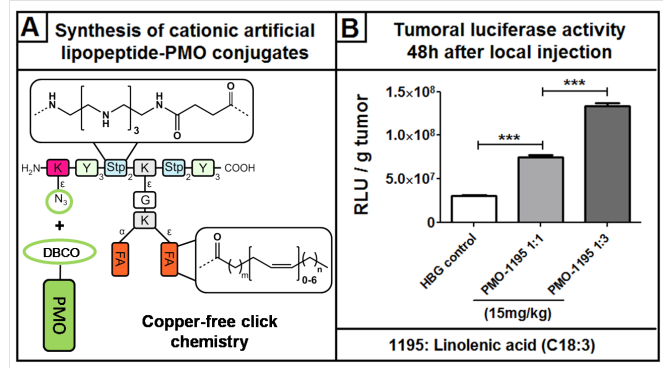
The manipulation of mRNA splicing by antisense oligonucleotides opens possibilities for the control of functional gene expression on a molecular level. It can be used to switch or modulate alternative splicing patterns in a therapeutic manner. Phosphorodiamidate morpholino oligomers (PMOs) are a class of artificial, uncharged nucleic acid analogs with favorable stability, nuclease-resistance, low immunogenicity and toxicity. Here, we present novel PMO conjugates with cationic lipopeptides for efficient delivery and splice-switching in tumor cells. The peptides were produced by solid-phase synthesis using the artificial amino acid Stp with precise oligoethylenimine-like structures,[1] α-amino acids and different hydrophobic modifications. Additionally, the lipo-oligomers were equipped with an azide functionality for the conjugation with dibenzocyclooctyne (DBCO) modified PMOs via click chemistry. The transfection efficiency of different conjugates was tested in a HeLa pLuc/705 reporter cell line containing a luciferase gene interrupted by a mutated human ß-globin intron with aberrant splice sites.[2] Successful delivery of splice-switching oligonucleotides into the cells induces correct splicing and restoration of the luciferase activity. After initial library screening of over thirty cationic oligomers, a particularly favourable T-shape topology was identified (Figure 1A). In a systematic variation of this lead structure, the type of contained fatty acids and their degree of unsaturation turned out to have critical impact on the kinetic and extent of splicing correction activity. An increase of the number of double bonds as well as a two-fold excess of free oligomer accelerated the onset of splice-switching activity. On a cellular level, the most active PMO conjugates induced an up to 150-fold increased luciferase expression compared to cells treated with HBG or free PMO at doses without distinct effect on cell viability. Effective splice-switching could also be achieved after intratumoral injection of the PMO-oligomer conjugates in murine HeLa pLuc/705 xenograft model. The treatments were well tolerated without observable adverse reactions and the beneficial effect of an oligomer excess was also verified in the *in vivo* situation (Figure 1B). The presented artificial cationic lipopeptide-PMO conjugates are considered to be promising candidates for therapeutic splice-switching with favourable activity/toxicity profile.



References:

[1] D. Schaffert, N. Badgujar, E. Wagner, *Organic Letters* **2011**, *13*, 1586-1589.

[2] S. H. Kang, M. J. Cho, R. Kole, *Biochemistry* **1998**, *37*, 6235-6239.

314. Fabrication and Performance of Size-Controlled CpG-Lipid Nanoparticles for Bladder Cancer Immunotherapy

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Bladder carcinoma is the most expensive tumor type to treat on a cost-per-patient basis from diagnosis to death. Treatment with Bacillus Calmette Guerin (BCG) instillations is the only approved immunotherapy in the clinic for treatment of superficial bladder carcinoma. Unfortunately, frequent relapses, high local morbidity, and a risk of systemic mycobacterial infection limits the long-term utility and effectiveness of BCG. It is well known that BCG utilizes

an adhesin protein known as fibronectin attachment protein (FAP) for binding to fibronectin (FBN) rich extracellular matrix of bladder tumor cells. Previously, we have shown that multivalent FAP-targeted liposomes promote FAP-FBN-Integrin microaggregation and internalization by a caveolae-dependent mechanism with a strict ≤ 70 nm size cutoff. Here, we report a microfluidic method for producing sub 70 nm lipid nanoparticles (LNPs) as a delivery system for CpG oligodeoxynucleotides as an immunotherapeutic cargo. CpG sequences have been shown to activate human plasmacytoid dendritic cells and B cells that expresses Toll-like receptor 9 (TLR 9) to mount an innate immune response characterized by the production of Th1 and proinflammatory cytokines. Since these receptors are located within intracellular acidic compartments, such as endosomes and lysosome, the delivery challenge is to formulate the negatively charged CpG oligonucleotides such that they reach the site of action in a biologically active state. Our in vitro data suggests that FAP-targeted LNPs specifically attaches to the cells present in the tumor microenvironment and become internalized. Then they get disassembled within acidic endosomal compartments to deliver functional CpG payloads in close proximity to TLR 9 which results in a tumor specific cytokine response. Thus, these size-tunable LNPs represents a potential translational platform for delivery of CpG oligonucleotides and an attractive alternative to BCG immunotherapy.

315. Optimization of Antisense Oligonucleotide Drugs for Skipping Dystrophin Exon 51 and 53 in Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a most common and fatal form of muscular dystrophy affecting 1 in every 3500 live male births. Becker Muscular Dystrophy (BMD) is the milder form ranging from almost asymptomatic to mild forms of DMD. Both result from mutations in the dystrophin gene. Exon skipping is one of the most promising therapies achievable in near future for the majority of DMDs. Identification of AOs with highest efficiency and specificity is therefore crucial for AO drug development. In this study, we have chosen to target 2 dystrophin exons, exon51, exon53 to search for the most effective morpholino (PMO) for the correction of the relevant DMD mutations in three systems of GFP report cells, normal human myoblast, and hDMD mice to screen 30 PMO drugs of maximal skipping potency. We found that 2 PMOs sequences targeting each exon 51 or exon 53 were able to skip the targeted exons with 30% or higher efficiency. Furthermore, we validated the optimized PMO sequences in patient-derived fibroblasts predicted to be suitable for therapeutic targeting of exon51, and exon53. We found that optimized PMO sequences showed skipping potency in all fibroblast cell cultures, but considerable variation is observed within the same subgroup of patient population. Finally, we further tested the murine sequence counterparts of the optimized AO drugs to skip exon51 and exon53 for histological, functional, and biomarker rescue in the mdxE52 mouse model. Our results provide standard screening procedure to identify effective AOs targeting human dystrophin exon for drug development to treat DMD patients.

316. Engineered U1 snRNPs Neutralize Deadly Splice Site Mutations Linked to Spinal Muscular Atrophy

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Spinal muscular atrophy (SMA) is caused by deletions or mutations of *Survival Motor Neuron 1* (*SMN1*) gene. *SMN2*, a nearly identical copy of *SMN1*, cannot compensate for the loss of *SMN1* due to predominant skipping of exon 7. However, correction of *SMN2* exon 7 splicing holds the promise for SMA therapy. Intronic splicing silencer N1 (ISS-N1) located immediately downstream of the 5′ splice site (5′ss) of exon 7 constitutes one of the major regulatory elements responsible for *SMN2* exon 7 skipping and an antisense oligonucleotide (ASO) that targets ISS-N1 is currently the only approved drug for SMA. The stimulatory effect of an ISS-N1-targeting ASO is ascribed to the displacement of the inhibitory factor(s) hnRNP A1/A2 and the structural rearrangement favoring recruitment of the U1 snRNP to the 5′ss of exon 7. Similar stimulatory effects on *SMN2* exon 7 inclusion have been observed employing engineered U1 snRNPs (eU1 snRNPs): the suppressor U1 with improved complementarity to the 5′ss of exon 7 as well as the U1 snRNAs that target downstream intronic sequences. A recently reported individual patient with severe SMA showed complete skipping of *SMN1* exon 7 due to a splice site mutation (G to C substitution at the first position of intron 7, G1C) that destroyed the authentic 5′ss. Here we employed eU1 snRNPs that promoted exon 7 inclusion in the context of G1C mutation by activating a strong cryptic 5′ss (N1-5′ss) located within ISS-N1. While suppressed in the presence of the authentic 5′ss of exon 7, the N1-5′ss is preferentially activated by U1 snRNPs targeting ISS-N1 as well as other sequences upstream and downstream of ISS-N1 in *SMN1* carrying G1C as well as other splicing mutation associated with different types of SMA. Our findings open up a new therapeutic avenue for patients with pathogenic splicing mutations in a critical exon, skipping of which causes SMA, one of the leading genetic causes of infant mortality.

317. Engineering HIV-1 Antibodies for Increased *In Vivo* Production Using DNA Encoded Monoclonal Antibody Technology

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The ability of HIV specific broadly neutralizing antibodies (bNabs) to protect or control HIV-1 is well-established in animal models. Though these antibodies are powerful and can prevent infection or control viral loads, the cost of production could severely limit the ability to use these antibodies in the field. Additionally, current vaccine approaches have failed to induce similar antibodies. We have developed a novel way to encode and produce bNabs *in vivo* using a synthetic DNA encoded monoclonal antibody (dMab) platform. Engineered plasmids encoding the heavy and light chain of over 20

HIV Envelope (Env) specific antibodies were produced and delivered intramuscularly to mice via electroporation with the CELLECTRA®-3P device. Initial production levels of many of the antibodies were low; however, using numerous optimization strategies including sequence, formulation, structural modifications and delivery, we can generate high levels (over 80ug/ml) of serum dMabs in mice. dMabs are detectable in the serum as early as two days after injection and are sustained for over half a year. Additionally, these antibodies retain functional capabilities including binding to Env and neutralization as measured by the TZM-bl neutralization assay. The *in vivo* produced dMabs have similar neutralization profile and potency as reported for the recombinant purified monoclonal bNabs. To lower the possibility of viral escape, using the dMab platform, we can encode and express multiple HIV-1 bNabs simultaneously in a single mouse. Furthermore, we are exploring the ability to encode and produce bispecific antibodies to increase potency and prevent escape. The dMab platform represents a novel technique for *in vivo* antibody production against HIV-1 and possibly other immune targets.

318. Identification of Novel Exon Skipping Targets in *Dysferlin* for Therapeutic Treatment of Dysferlinopathy

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Background: Dysferlinopathy is a progressive myopathy caused by mutations in the *dysferlin* (*DYSF*) gene. Dysferlin protein plays a major role in plasma membrane resealing. Some patients with large *DYSF* deletions have shown remarkably mild symptoms, suggesting some regions of *DYSF* can be removed without significantly impacting protein function. Antisense-mediated exon skipping therapy uses synthetic molecules called antisense oligonucleotides (AOs) to modulate splicing, allowing exons harboring or near genetic mutations to be removed ("skipped") and the open reading frame corrected. Previous studies have focused on *DYSF* exon 32 skipping as a potential therapeutic approach, based on the observation of a mild phenotype associated with the in-frame deletion of exon 32. To date, no other *DYSF* exon skipping targets have been identified and the relationship between *DYSF* exon deletion pattern and protein function remains largely uncharacterized. In this study, we identified novel *DYSF* exon skipping targets by utilizing a plasma membrane wounding assay to test the effectiveness of plasmid constructs carrying mutant dysferlin at rescuing membrane resealing in dysferlinopathy patient cells. **Methods**: We performed site-directed mutagenesis on plasmid constructs containing full-length *dysferlin* to generate *dysferlin* plasmid constructs with various exons deleted. These plasmid constructs were then transfected into dysferlinopathy patient cells and cells were subjected to a membrane wounding assay. We then designed AOs corresponding to our *dysferlin* exon deletion constructs and transfected them into patient cells, subjecting the cells to a membrane wounding assay. **Results**: Plasmid constructs lacking *DYSF* exons 19-21, 20-21, and 46-48 were unable to rescue plasma membrane resealing *in vitro*, while constructs lacking *DYSF* exons 26-27 and 28-29 were able to rescue plasma membrane resealing. AOs targeting *DYSF* exons 28-29 were able to facilitate efficient exon skipping *in vitro*. **Conclusion**: We have identified two novel exon skipping targets in *dysferlin*: exons 26-27 and exons 28-29. Successful translation of these findings into the development of clinical AO drugs would establish new therapeutic approaches that would be applicable to approximately 15% of dysferlinopathy patients worldwide.

319. Identification of Circulatory miRNAs in Canine Cancer Patient Liquid Biopsies

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Cancer is the second leading cause of disease in United States (WHO and CDC), with 591,700 reported deaths in 2014 and estimated 600,920 deaths in 2017. Currently, cancer screening is invasive and is limited to clinical presentation, radiology, biochemical tests, and pathological analysis of tumor tissues. Most of these cancers are un-detectable at an early stage and are often diagnosed after metastasis. There are no or few diagnostic markers or tests available for most types of cancer. Whatever is available, is generally focused on a single tumor type. Improved cancer detection, especially early in the disease process, and preferably performed non-invasively, would potentially improve cancer patients' health care. Blood-based liquid biopsies hold promise in this area. In recent years, circulating micro RNAs (miRNA) have been identified as potential cancer biomarker as they are stable in blood and are protected from endogenous RNAse activity. miRNAs are small, 19-25 nucleotide long, non-coding RNA molecules found in cells that play an important role in post-transcriptional regulation of gene expression. These miRNA are found inside the cells, however a portion of them are shed into the circulation in lipid coated particles known as exosomes. We have recently identified a series of exosomal miRNAs whose expression is altered over a wide range of canine tumor cell lines. Exosomal micro RNAs (miRNAs) from 13 different cancer cell lines and normal canine fibroblasts were collected, sequenced and quantitated. miRNA sequences were referenced to the canine miRNA database and each miRNA expression or read count was normalized to the total read count of the samples. Fold differences were calculated for each miRNA by comparing normalized expression of miRNAs in the cancer cell lines to normal canine fibroblasts cells. Based on this data, a list of miRNAs that were specifically altered in all or almost all of the canine cancer cell lines, in comparison to normal canine fibroblasts, was generated. We are currently validating the pan-tumor specificity of these previously identified miRNAs in clinical canine cancer patients. We have collected heparinized plasma from the Auburn University Clinical Pathology Laboratory from patients with and without a cancer diagnosis. Our non-cancer patients are a mixed pool of different diseases and are divided into two groups of patients: young (≤4 years of age) patients and geriatric (≥8 years of age) patients. Our cancer patients are a mixed pool of different cancer diagnoses. The plasma is processed for isolation of exosomal RNA and quantitative PCR is performed to compare the expression of our previously identified miRNAs between patients with cancer and those without tumors. Validation of pan-tumor specific altered miRNAs in

canine cancer patients will provide us with biomarkers to screen for tumor. Such markers could be used to screen at-risk individuals for the presence of cancer long before symptoms appeared and would allow tumors such as ovarian and prostate cancer to be identified much earlier than currently possible.

320. cGMP Synthetic Gene Synthesis to Enable Personalized Medicine

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Advances in genome sequencing and informatics are integral to the development of personalized therapies for a variety of diseases. For example, deciphering patient and tumor-specific genomic and clinical details shows great promise for the development of personalized medicine treatments using synthetic DNA constructs that target cancer neoantigens. Historically, the creation of synthetic genes has been performed in a research use only (RUO) facility. While RUO laboratories enable research that can lead to the discovery of promising personalized medicine treatments, they are typically not appropriate for the transition of a therapy toward the clinic. As a project moves from a research phase, to a pre-clinical phase, to clinical trials, it is important to establish properly documented and robust processes that will ensure consistent quality and patient safety. To facilitate the transition of personalized treatments from bench to bedside, SGI-DNA has established a cGMP production laboratory for synthetic DNA constructs. These additional capabilities extend and complement our labs established processes for creating research-grade synthetic DNA. Our ability to rapidly produce DNA constructs under a documented cGMP process is designed to address the requirements of regulatory authorities and more efficiently move a project from a research to a clinical phase. Here, we describe the characteristics of a cGMP gene synthesis facility and discuss creation of cGMP constructs targeting cancer neoantigens as part of a preclinical/clinical program.

Vector and Cell Engineering, Production or Manufacturing I

321. Widespread Transduction and Spread of a Modified AAV2 Capsid in the Non Human Primate Central Nervous System

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Background: Achieving global transduction in the Central Nervous System (CNS) with adeno-associated viral (AAV) vectors is an important goal in the treatment of a number of neurological disorders. We have addressed this issue with a modified AAV2 vector (termed AAV2-HBKO), which is incapable of binding to the heparan sulfate proteoglycan (HSPG) receptor. **Objective:** The goal of this study was to evaluate the efficiency of CNS transduction of AAV-HBKO encoding green fluorescent protein (GFP) in non-human primate (NHP) after either thalamic, intracerebroventricular (ICV) or a combination of thalamic and ICV delivery. **Results:** Thalamic injection resulted in widespread transduction of neurons in deep cortical layers, and several subcortical regions, indicative of robust bidirectional axonal transport. AAV2-HBKO also underwent anterograde transport to transduce motor neurons in the spinal cord via the corticospinal tract. ICV delivery similarly resulted in widespread cortical transduction, with the striking distinction that oligodendrocytes within superficial layers of the cortex were the cell type primarily transduced. Robust motor neuron transduction was also observed in all levels of the spinal cord with ICV delivery. The combination of thalamic and ICV delivery resulted in transduction of all cortical layers; with oligodendrocytes transduced in the superficial layers and neurons transduced in the deep layers. Several subcortical regions were also transduced, indicative of bidirectional axonal transport. **Conclusion:** Our results demonstrate that a variant AAV2 vector, unable to bind its HSPG receptor, has the ability to spread via retrograde and anterograde transport and that the route of delivery impacts cellular tropism. The combination of thalamic and ICV delivery allows for global CNS transduction.

322. Preclinical Evaluation of ZAPR™ GMP Red Blood Cell Lysis Buffer for Use in Manufacturing CD34+ Stem Cell Derived Gene Therapy Products

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Lentiviral vector (LVV) transduction of CD34+ hematopoietic stem and progenitor cells (HSPCs) holds tremendous promise as an *ex vivo* gene therapy approach. LVV-modified HSPCs are currently being evaluated in multiple clinical trials targeting hemoglobinopathies, such as sickle cell disease (SCD) and β-thalassemia. Despite their genetic similarity, SCD presents additional hurdles in optimal cell product manufacturing. For example, it is known that SCD red blood cells (RBCs) can bind peripheral blood (PB) mononuclear cells (PBMCs) to a significantly greater extent than healthy donor (HD) RBCs (Chaar V, et al. Haematologica 2010). Here, we show that, using SCD bone marrow (BM) as the CD34+ HSPC source, RBCs are co-purified with the desired HSPCs, and investigate the use of a GMP grade RBC lysis buffer, ZAPR™, for removal of residual RBCs from the cell product manufacturing process for SCD BM. Flow analysis of enriched PBMCs from SCD BM showed that approximately 20-40% of cells were RBCs, with 3-7% of cells staining positive for both CD45 and glycophorin A, indicating a PBMC-RBC aggregate. By comparison, a typical HD PBMC enrichment would be expected to have <3% RBCs. Incubation of SCD PBMCs with ZAPR™ per manufacturer protocol reduced RBC contamination to approximately 3-6%, and the PBMC-RBC aggregation to <3%. To assess the tolerability of the RBC lysis protocol, SCD and HD whole blood samples were incubated with ZAPR™ for longer than the recommended 10 minutes (10-120 minutes) prior to washing and plating for methylcellulose colony formation. Prolonged exposure to ZAPR™ had no impact on colony formation. The clinical utility of ZAPR™ was investigated at scale by splitting a HD mobilized PB product in half and processing one half with ZAPR™ treatment prior to CD34+ cell enrichment while the control arm was CD34+ enriched without RBC lysis. ZAPR™ treatment resulted in higher CD34+ cell yields and purities compared to the control arm, 78% versus 66%, and 96% versus 87% from control and ZAPR™ arms, respectively. This resulted in a total process recovery of 72% in the ZAPR™ arm compared to 58% in the control arm. Next, both CD34+ arms were transduced with LVV. The resulting products had comparable vector copy numbers (VCNs) for the control and ZAPR™-treated arms, 3.1 copies/diploid genome (c/dg) and 2.8 c/dg, respectively, as well as similar percent markings, 80% and 74%, respectively. Cells were transplanted into busulfan-conditioned immunocompromised mice (NSG) in limiting dilution (1×10^4 to 3×10^5 cells/mouse). Four months post-transplant, BM from NSG mice was analyzed for engraftment of long-term repopulating cells. There was no statistically significant difference ($p=0.14$) in overall engraftment between cells treated with and without ZAPR™, 22.5%±2.8% and 29.1%±3.3%, respectively (measured by the presence of human CD45+ cells in the NSG mice receiving the highest dose of CD34+ cells). In addition, there was no significant difference in the multilineage potential of the engrafting cells. VCN analysis revealed no significant difference in engraftment of transduced cells. The frequency of engrafted long-term repopulating cells was calculated to be 1: 50,432 (95% confidence interval 1:87,011 - 1:29,230) and 1:41,418 (95% confidence interval 1:72,756 - 1:23,578) for the control and ZAPR™-treated arm, respectively. Taken together, these data suggest that ZAPR™ may be applied to the manufacturing process for *ex vivo* CD34+ stem cell-derived gene therapy products when needed to decrease contaminating RBCs without affecting the viability or recovery of PBMCs, and more importantly, without adversely affecting the critical long-term repopulating CD34+ cells.

323. Enhancing shRNA-Containing Lentiviral Vector Production for Gene Therapy

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Hematopoietic malignancies are characterized by an inability of the immune system to function correctly and are fatal if left untreated. Treatments to date include bone marrow transplants and in some cases like Human Immunodeficiency Virus (HIV)-lymphoma patients, lentiviral vector therapy have been used to genetically-modify transplant cells in order to also protect these patients from HIV infection. These lentiviral vectors can be used to express artificial short-hairpin ribonucleic acids shRNAs that target one or more target genes of choice. These combinations are varied but contain at least one promoter driving multiple shRNAs or multiple promoter-shRNA pairings in one vector. Besides these vectors, single endogenous intronic miRNA (mirtron) gene cassettes have been used to expresses multiple shRNAs from the same promoter. Various combinations of these shRNA vectors have been used in clinical and pre-clinical studies for gene therapy. Although efficacious in target knockdown, clinical-scale production of shRNA-containing vectors has been a known hurdle in the gene therapy world. During vector production, shRNAs can be processed by the endogenous miRNA processing machinery and then go on to target the original vector and subsequent viral particles produced. This process lowers overall intact viral particle count and increases the cost of large scale viral production. Along with vector targeting, mirtrons are sensitive to alternative splicing, which can yield vector strands of varying length that may not function correctly in target cells. To address these concerns, we have chosen to use splice-inhibiting antisense oligonucleotides (ASOs) to protect mirtron vectors during packaging. In addition to this, we have chosen to knockdown Ago2, the main catalytic protein of the miRNA processing machinery, during packaging in order to increase single promoter-shRNA paired vector production. The subsequent viruses produced were used to determine viral titers, transduction efficiency, and target knockdown. Although both methods showed increased viral production, splice-inhibiting ASOs showed a greater increase in titer improvement. The results from this study indicate that clinical lentiviral production of shRNA-containing vectors would benefit from customized vector production modifications.

324. Novel Constitutive Producer Cell Line & GMP Compatible Dowstream Process for Lentiviral Vector Manufacturing

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Background The use of lentiviral vectors in gene therapy clinical protocols has increased substantially in the past 5 years. The approval of several gene therapeutic products in USA and Europe, as the ADA-SCID treatment Strimvelis, and the very encouraging results obtained with chimeric antigen receptor (CAR) T cells therapy followed by the very recent approvals of Kymriah and Yescarta, have boosted the demand and quality of gene therapy vectors. Lentiviral vectors have been progressively undertaking gammaretroviral vector use in ex-vivo gene therapies. However the knowledge on gammaretrovirus, particularly its manufacturing, is far more mature. While the production of gammaretrovirus rely on stable producer cell lines and perfusion systems, enabling high cell density and longer term productions, most of the bioprocesses for lentiviral bioproducts rely on transient transfections and short term batch productions. Dowstream processes remain a challenge for both vectors due to their low stability, requiring fast purification processess. **Experimental approach** At the upstream process, many of the challenges lentiviral bioproducts present in their manufacturing are related to the apoptosis-leading cytotoxicity of some of the vector components. Supported on our long track experience and enabling tools developed for gammaretrovirus manufacturing, we undertook the challenge of establishing a constitutive stable lentiviral producer cell line. To adress this challenge we reduced the cytotoxicity of the lentiviral vector expression components. Several strategic novelties were introduced in the dvelopment of the cell line namely: (i) the use of a mutated gag-pro-pol, (ii) introduction of all the third generation lentiviral expression cassettes by chemical transfection and (iii) performing only one clone screening step. After establishing a stable producer cell line the culture conditions were developed with the main aim of extending bioreaction culture time and viral vector total yields. At the downstream process lentiviral vectors face the challenges common to retroviridae family of vectors namely short half-lives at room temperature, sensitivity to pH variations, salt concentrations and shear stress. The purification strategy developed was based on disposable and easily scalable technologies. A final concentration achiving 10^8 TU mL^{-1} was targeted since the concentration step iself allows to reduce the impurities on the process and improve the transduction efficiency. **Results and discussion** A lentiviral producer cell line constitutively producing infective titers above 10^6 TU.mL^{-1}. day^{-1} was established. Moreover the new protocol to generate the cell line enabled its developmet in less than six months. The cell line showed to be stable, consistently maintaining vector productivity over one month in the absence of antibiotics. At the bioreaction process it was possible to maintain the cells continuously producing over 10 days. At downstream a scalable protocol for lentiviral vectors that is easly to transferable to a GMP environment was developed, combining microfiltration, anion-exchange, and ultrafiltration membranes technologies maximizing infectious LVs recovery, allowing generation of clinical-grade viral vectors without the need for cleaning validation in a cost-effective manner. The results to be presented will qualify the strengths and advantages of these strategies.

325. Dual Tangential Flow Purification Improves Downstream Processing of Large Scale Clinical-Grade Preparations of Lentiviral Vectors for Phase I/II Clinical Trials

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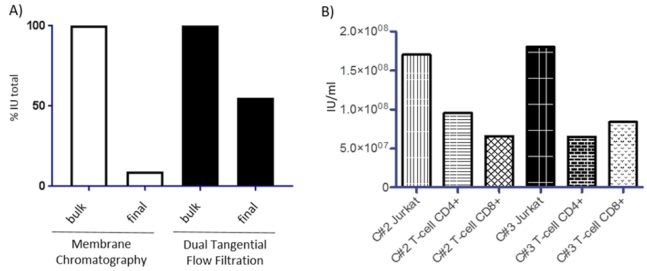
Lentiviral vectors (LVs) are currently the most widely used vector system for hematopoietic stem cell (HSC) gene therapy and immunotherapy applications. With advances in vector design and large-scale production, LVs have become a safe and effective gene delivery system for many clinical applications. However, large-scale clinical-grade production using current good manufacturing practice (cGMP) is costly and often associated with significant vector loss during the manufacturing, especially when membrane chromatography (MC) based purification is used (Figure A). To overcome this limitation, we implemented a semi-closed production system based on mammalian cell transfection followed by downstream purification and concentration methods with minimal use of filtration and omission of membrane-binding steps as well as centrifugation. We developed a modified process using two tangential flow filtration (TFF) steps "in tandem" instead of MC and compared our process to the traditional MC-based method. Vector production was initiated by transient transfection of 293T cells with a four-plasmid system using calcium phosphate. Vector containing media (VCM) was harvested from cell stacks at two time points post transfection. Clinical grade processing was either done by Mustang Q anion exchange-based chromatography followed by Benzonase treatment and final concentration by single TFF, or with a dual TFF approach with initial twentyfold concentration after which the reduced VCM was treated with Benzonase. Following the Benzonase treatment a TFF-based media exchange was performed, and vector concentrated. Both large-scale clinical-grade preparations produced with this new platform based on dual TFF purification resulted in cGMP grade functional product with a 2-fold loss of produced vector during purification and final titers in the mid 10^7 range. In contrast with the MC-based approach, while the final titer was comparable, the total loss of functional particles was 10-fold (Figure A). Clinical grade vector from both dual TFF manufacturing runs was tested on Jurkat T lymphocyte cells, where they achieved high transduction efficiency that translated to titers in the 10^8 infectious units per milliliter (IU/ml) range (Figure B). High transduction efficiency was also observed in CD4+ and CD8+ activated human T-cells where the vector showed low toxicity and stable transgene expression (Figure B). Due to these

findings in T-cells, both cGMP vector preparations purified with the dual TFF platform qualified to be subsequently utilized in phase I/II clinical trials. In summary, we developed a protocol for clinical-grade lentiviral vector production with improved total vector recovery. The final product passed cGMP quality tests and qualified for use in clinical trials. A dual TFF-based platform can allow for reduction of volume, manufacturing effort, and thus help saving cost.



A) Comparison of vector loss during purification using either membrane chromatography (white bars) or dual tangential flow (black bars) after bulk production. **B)** Functional titration after transduction of T-cell line and human T-cells with cGMP vector purified by dual tangential flow filtration.

326. Feasibility of Cryopreserved Mobilized Peripheral Blood as a Starting Material for *Ex Vivo* CD34+ Stem Cell Gene Therapy Products

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Mobilized peripheral blood mononuclear cells collected by apheresis, referred to as hematopoietic progenitor cell-apheresis (HPC-A), are a starting material for autologous gene therapy cell product manufacturing. Generally, multicenter clinical trials ship fresh HPC-A collections to centralized manufacturing facilities. Due to variability in mobilization and collection, multiple collections may be necessary and therefore, processing typically begins 24-48 hours post collection. Coordination of the HPC-A collection, shipment, and processing of the fresh collections prior to expiration poses considerable logistical challenges. A potential solution is the use of cryopreserved HPC-A starting material. Frozen HPC-A would provide considerable flexibility with respect to when cell processing could initiate, enabling efficiencies in manufacturing scale-up, and potentially facilitating global access to these therapies. Additionally, the cryopreservation process could improve the uniformity of the starting material by depleting contaminating cell types and eliminating the possibility of variable and uncharacterized cellular interactions that can occur between collection and cell product manufacturing. We report here progress toward the use of cryopreserved HPC-A starting material for the generation of lentiviral vector-transduced CD34+ cells. To this end, we have developed an automated functionally closed manufacturing platform for cryopreservation of HPC-A, based on an established and robust blood processing technology, that is readily transferrable to a clinical site. Using HPC-A collected from 3 healthy donors and split for processing at clinical scale, frozen HPC-A yields higher viable CD34+ cell recovery and purity post enrichment compared to fresh HPC-A, 76.7%±0.20 compared to 60.6%±0.25 recovery, and 94%±0.04 compared to 92.3%±0.06 purity, respectively. To compare the processes side-by-side, we transduced two batches of CD34+ cells from a single donor using fresh or frozen HPC-A as the starting material, and the respective cell products were evaluated in an immunocompromised (NSG) mouse transplant model. The cells (pre-transplant) had similar vector copy numbers (VCNs): 3.7±0.1 copies/diploid genome (c/dg) for the fresh cells, and 4.1±0.4 c/dg for the frozen cells, with >80% of cells transduced. NSG mice were conditioned using busulfan and transplanted with $1x10^6$ CD34+ cells per recipient, and bone marrow (BM) was analyzed at 8 (short-term) and 16 (long-term) weeks post-transplant for human cell chimerism and engraftment of transduced cells. At both time points, there was no statistically significant difference in engraftment of human cells in the mouse BM, and engrafted human cells retained multilineage differentiation capabilities. Transduction of long-term engrafting cells was further quantified by performing individual colony VCN analysis on human cells isolated from NSG BM. Both the fresh and frozen HPC-A arms had similar median VCNs of approximately 2 c/dg with 70% and 66% of colonies transduced, respectively. Taken together, these data suggest that both fresh and frozen HPC-A can be used as starting material in the manufacturing process for *ex vivo* gene therapy CD34+ cell products without affecting the viability, recovery, or transduction efficiency of CD34+ cells. Importantly, long-term engraftment of transduced cells and the multilineage potential of engrafted cells is retained, demonstrating the additional cryopreservation step does not negatively impact this critical feature of hematopoietic stem and progenitor cell function.

327. A Simple and Robust Process to Scale-Up Lentiviral Vector Production by Transient Transfection of Suspension Cell Culture in Stirred-Tank Bioreactors

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Lentiviral vectors (LVs) are powerful tools for cell therapy because of their capacity to efficiently integrate their payload into the chromosomes and thus stably modifying the targeted cells. One of the most popular methods to produce LV is by transient transfection of mammalian cells with plasmids encoding the vector components. Using a clone of HEK293 cells (HEK293SF-3F6) that was adapted to serum-free suspension culture, and for which a cGMP master cell bank is available, we have developed a simple process to produce LV by transient transfection using polyethylenimine (PEI) as a transfection reagent. In the present study, we demonstrate scalability of this process by producing a third generation LV encoding a chimeric antigen receptor (LV-CAR) using a mix of four different plasmids. Through

a design of experiments (DOE), we first optimized the total DNA concentration, the DNA/PEI ratio, and the concentration of sodium butyrate in 125-mL shake-flask cultures with 20 ml working volume each. The cells at 1.0E+06 cells/mL density were transfected in a serum-free medium at a fixed plasmid ratio using PEI-DNA polyplexes. The infectious titer of LV, harvested at 3 days post-transfection, was measured by flow cytometry after transduction of HEK293A cells using an antibody raised against the expressed protein of the gene product (CAR). The best transfection conditions were then validated in larger 1-L shake-flask cultures with 175 mL working volume. The optimal transfection conditions dictated to use low concentration of DNA (0.3 μg/ml), a DNA/PEI ratio of 1 and 5 mM sodium butyrate which resulted in a titer of 2.7E+07 transduction units (TU)/ml of culture, for a specific productivity of 27 TU/cell. Using these optimized conditions, production was then scaled-up in a 3.5-L stirred-tank bioreactor at a 2.6 L working volume and then in a 200-L single-use stirred tank bioreactor at a 70 L working volume. The infectious titer measured in the culture medium at 3 days post transfection was 1.6E+07 and 1.5E+07 TU/ml for the 3.5-L and 200-L bioreactors, respectively. This study demonstrates that the simple transient transfection process that we have optimized to produce LV using our clone of HEK293SF cells is robust, scalable and amenable to cGMP manufacturing.

328. Establishment of a Scalable Manufacturing Platform for *In-Silico* Derived Ancestral Adeno-Associated Virus Vectors

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Adeno-associated virus (AAV) is an effective viral vector for gene therapy due to its non-pathogenicity, high transduction efficiency, broad tissue specificity and long-term transgene expression. The clinical use of AAV vectors, however, pose a significant challenge - pre-existing immunity (PEI) is observed in up to 70% of the people who have had prior exposure to wild-type AAV, thereby rendering treatment ineffective and excluding the majority of patients from such therapies. In addition, large-scale production of AAV vectors is labor-intensive, and results in high production costs. Targeting these challenges, we licensed a novel synthetic ancestral AAV vector (Anc80) that is resistant to PEI, and developed a high transfection efficiency suspension HEK293 cell line that would predominantly reduce biomass load as well as costs associated with commercial manufacturing, created an effective, cost-efficient, scalable manufacturing process to meet potential market needs. We isolated and identified highly-transfectable suspension HEK293 clonal cells via flow cytometry, limiting dilutions and subsequent single cell expansion into agitated cultures. We specifically selected one "super-clone" with productivities in the range of 2~3E+04 vg/cell for Anc80, and 0.9-1.0 E+05 vg/cell for AAV8-comparable or better than other available HEK293 suspension cell lines. A process that was optimized for media and production cell densities was successfully scaled up to a 50L in a stirred tank bioreactor, while maintaining the productivities. This cell line was further characterized for stability, identity and other routine testing and will be available as a completely traceable GMP cell bank. Therefore, this high producer HEK293 clone is an excellent candidate for the clinical and commercial production of Anc80 and other serotypes of AAV.

329. Approaches to Ameliorate the Amplification Process of High-Capacity Adenovirus Vectors

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High-capacity adenovirus vectors (HCAdV) are devoid of all coding genes and are a powerful tool to deliver large DNA cargos into human cells. The vectors are amplified by rescuing a transfected vector genome by means of a helper virus (HV) in producer cells. However, their implementation as therapeutic vectors appeared challenging and lately less innovation emerged in this field compared to other viral vectors. One of the major hurdles is the lack of a robust production process in terms of HCAdV concentration and HV contamination, to constantly provide high-quality vector batches. In a sequential system like the HCAdV production every parameter can lead to a synergistic effect with great impact on the outcome. In this study we present a collection of approaches to improve the production process in order to push the development of HCAdV as therapeutic vectors. One aspect of improvement was the initial transfection and infection step for virus rescue. The established schedule is to transfect cells with the linearized HCAdV genome prior to HV transduction. We found in a dual fluorescent reporter experiment that the transduction of transfected cells is less efficient than of untransfected cells. Furthermore, during the vector amplification procedure the transfection and transduction of the producer cells was enhanced by applying the HV at the same time as transfection of the HCAdV vector genome. The next aspect is that there is evidence that the expression of transgenes affects the amplification efficiency of vectors. This phenomenon was analyzed quantitatively using constructs which carry transgenes with different expression profiles, e.g. due to tissue-specificity of the promoter. Our data revealed that a down-regulated transgene can lead to several hundred-fold higher vector genome copy numbers after serial amplification compared to the transfected input HCAdV DNA molecules. Constructs bearing constitutively transcribed transgenes only reached a level comparable to the transfected input. Finally, the accurate characterization of produced vector batches in terms of viral particles and infectious particles is a highly discussed topic. Here we analyzed data acquired by multiplexed quantitative PCR to provide improved means for vector batch description including accurate molecular ratios of HCAdV and HV and their ability to transduce human cells. Analysis was optimized to run in a single reaction and yielded highly correlated data. We are convinced that the presented concepts will provide important steps towards an improved HCAdV vector production procedure to allow the therapeutic use of HCAdV vectors in the future.

330. Variability in AAV Genome Quantification is Reduced with Inclusion of the Non-Ionic Surfactant Pluronic F-68 across Serotypes and qPCR versus ddPCR

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Quantification of recombinant adeno-associated viruses (AAV) for gene therapy is routinely performed by measuring packaged AAV genomes using specialized polymerase chain reaction (PCR) technologies, either the standard-based quantitative, real-time PCR (qPCR) or, more recently, the standard-free droplet digital PCR (ddPCR) methods. Both platforms offer the possibility of high specificity, high sensitivity absolute measurement of recombinant viral genomic DNA, with ddPCR generally accepted as the most precise and sensitive. Notwithstanding these features, however, accuracy of AAV quantification remains challenging, for example, with a reported inter-laboratory variation as high as 100-fold. Accuracy and standardization of vector quantification is essential for comparing AAV manufacturing processes and quality control of vector lots, especially toward vector potency and safety, and ultimately gauge dosage of research and clinical subjects across sites. We report here that inclusion of the non-ionic surfactant Pluronic™ F-68 in qPCR or ddPCR protocols dramatically increases AAV genome detection in a serotype-dependent manner by minimizing affinity of the virus for plastic surfaces. Equally important, Pluronic™ F-68 affirms the expectation that qPCR and ddPCR can be used interchangeably for the accurate quantification of high copy number samples, such as are routinely encountered in an AAV production facility. We found that accuracy is improved and validated by performing simple cross-referencing steps at the level of qPCR standard preparation and the inclusion of Pluronic™ F-68 in the qPCR standard/AAV sample diluent. Having implemented these measures, quantification of single-stranded AAVs agree to within 10-30% across qPCR and ddPCR platforms, underlining their equivalence. Caveats aside, our results indicate that a consideration of the biophysical properties of the AAV capsid is important for accurate AAV quantification in addition to the choice of methodology for this assessment.

331. CAP-GT, a Scalable Platform for Transient Recombinant AAV Vector Production in Human Suspension Cells

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Gene therapy has been long fascinating because of its potency to treat a disease at genetic level. Up to this day, recombinant adeno-associated virus (rAAV) based vectors have become a preferred tool to deliver genes of interest (GOI) as seen by the use of these vectors in numerous gene therapy clinical trials. Nevertheless, rAAV vector production to this date still faces bottlenecks concerning host cell productivity and scalability. Although transfection-based large-scale production methods have been developed, further improvements are still required to increase vector yields and vector quality in order to supply vector genome doses required by later stage clinical phases. With our CAP and CAP-T cell lines, we have developed the CAP-GT platform for transient rAAV production in high density suspension culture. To optimize the production of rAAV vectors in this platform, we applied different approaches for protocol optimization and process development with PlasmidFactory's 2-plasmid-system, using e.g. the ambr® system. In a DoE approach, we could identify conditions for high titer transient rAAV vector production in both, CAP and CAP-T, cell lines. Scale-up using these optimized conditions resulted in a consistent and high titer vector yield from the CAP-GT platform in a single-use bioreactor system.

332. Abstract Withdrawn

333. Improving Oncolytic Adenovirus Manufacturing: The Effect of Alternative Detergent to Triton on Downstream Process

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Oncolytic virotherapy represents a new class of cancer drugs that can be used to improve the traditional treatments. Adenovirus is a well-known and extensively characterised oncolytic agent. The rise of the number of clinical trials using this virus generates the demands to develop an improved purification platform to lower costs of treatments. In phase I and II clinical trials, six unit operations are often utilized for adenovirus purification at 10-50L scale: cell lysis and nuclease treatment; clarification; ultrafiltration; intermediate purification and polishing. The use of Triton X-100 detergent in the lysis step is a popular approach, since it is easily scalable and well described. However, Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation prohibits its use, without authorisation, from 4 January 2021. In this work, we report an improved oncolytic adenovirus purification process for phase I and II clinical trials. We proposed a 6-steps process using state-of-the-art materials, such as high-throughput Capto Q ImpRes media for intermediate chromatography purification. We also investigated the use of Polysorbate 20, for cell lysis and its effect in product recovery and impurities removal throughout all the steps of the purification process. Overall, over 40% of total and infectious particles was recovered and 99% of the impurities (host cell protein and dsDNA) were removed. Additionally, we will present a case on the use of Polysorb 20 as a replacement for Triton X-100 during cell lysis. Product recovery, potency, purity and the effect of manufacturing holding points will be discussed.

334. Optimization of a Monolithic Chromatography-Based Manufacturing Process for Adenoviral Vectors

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We have recently established an Adenovirus purification method by anion exchange chromatography using a Polymethacrylate CIMmultus monolithic column functionalized with quarternary ammonium groups (BIA Separations). Monolithic columns possess a uniform, continuous network of large-sized pores and channels that are suited for the purification of large biomolecules such as virus particles. Compared to conventional packed-bed resins, monolithic columns allow more effective mass transfer which leads to reduced virus processing time. Gradient and step elution patterns were tested using Benzonase-treated and clarified adenovirus infected 293 cell lysates purified on a 1 mL CIMmultus column. A step elution method was identified which resulted in up to 47% virus recovery and over 90% removal of total protein content in the purified virus fractions. Scale up of this step elution method using an 8 mL CIMmultus column resulted in the same sharp virus peaks observed on the 1 mL column. Further work will focus on increasing yield and purity by incorporating a gradient elution step to separate contaminant proteins. We will also utilize Adenovirus cell lysates prepared from an A549-based cell line (SF-BMAdR) that expresses the Adenovirus E1 region (Gilbert *et al*, J. Virol Methods 208, 2014). Host cell proteins and host cell DNA present in the purified virus fractions from 293 or SF-BMAdR cells will be assessed and total virus recovery and yield will be determined.

335. Development and Validation of Droplet Digital PCR Assay for Viral Vectors Used in Cell and Gene Therapy

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Droplet Digital PCR (ddPCR) is a powerful technology which combines the principles of standard PCR and QPCR to perform absolute quantitation of a test sample. Advantages of using Droplet Digital over conventional QPCR include greater accuracy and precision of quantitation, end-point instead of real-time analysis, higher signal to noise ratio for decreased inhibition, and the ability to quantitate without the use of a standard curve. Accurate quantitation of vector genome copies is critical for viral gene therapy products. Several variations of the method have been developed for accurate titration of the most commonly used Viral and Gene Therapy Vectors: Adenovirus, Adeno-associated virus (AAV), Retrovirus, and Lentivirus. To accommodate the variety of products existing in every viral vector category, primers/probe sets have been designed to target common regions present in every viral genome type. All assays have been optimized to reduce sample manipulation steps leading to ddPCR and are fully validated to demonstrate specificity, accuracy, intermediate precision, repeatability, linearity, range and limit of quantitation. The methods may be customized for specific vector applications. Combined with an automated platform, results may be obtained within 1 day allowing for important process development, manufacturing, and patient dosing decisions to be made with the most accurate data that the industry has to offer. As applications continue to grow with this emerging technology, additional offerings for copy number variation and to monitor viral integration for Lentivirus and Retrovirus applications are in development.

336. Comparing Multi-Layer Vessels for Large-Scale Lentiviral Vector Production

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Lentiviral (LV) vector-mediated gene delivery protocols have shown great clinical promise in the gene and cell therapy field. LVs offer a high rate of transduction into many different cell types, the ability to transduce non-dividing cells, stable gene integration, and reduced risk of insertional mutagenesis when compared to gamma-retroviruses. However, the production and purification of high-quality LVs for clinical trials is challenging. Herein we compare different disposable multi-layer platforms from Corning and Greiner Bio-One for the scale-up and production of high titer LV. Following LV harvest, particles were concentrated and functional titer, p24 levels, and host cell protein expression was analyzed. The common research laboratory strategy for packaging LV vector is a plate-based culture method. We routinely generate LV stocks of 10^8-10^9 transduction units (TU) per ml using such plate-based methodologies. We compared Corning HYPERflask®, Corning HYPERstack®, and Greiner Bio-One CELLdisc™ vessels. All 3 culture vessels generated LV titers similar to our small-scale plate-based methods (>$1.0x10^8$ TU/mL). A p24 ELISA also revealed similar values from the 3 culture vessels (10^6-10^7 pg/mL). The HYPERflask® had increased host cell protein (HCP) contamination, yielding >$1.0x10^4$ ng/mL HCP in comparison to $1.8x10^3$ ng/mL HCP from plate-based cultures. The CELLdisc™ and HYPERstack® contained reduced HCP and were similar to the plate-based method. Each of these multi-layer platforms have a niche in LV manufacturing. The CELLdisc™ covers a range of cell culture surface areas, with larger sizes possibly requiring the use of the CELLswing™ for automation. Also, reduced HCP contamination can lower downstream processing costs. The HYPERflask® is easily maneuverable and excellent for laboratory size preparations, but would require high labor input to process the appropriate volume for large clinical-grade preps. The HYPERstack® cell culture vessels feature a large cell growth area, a closed system approach with flexible tubing for liquid handling, the ability to connect multiple vessels, and is manually maneuverable. Each of these disposable multi-layer platforms were easy to use, and produce high titer LV suitable for early stage-scale clinical LV production.

337. Development of a Scalable AAV Harvest Clarification Process

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For large scale production of Adeno-associated Virus (AAV), a scalable process to harvest the viral vector from suspension culture is needed. Usually, AAV from adhesion culture is usually clarified using inert filters due to its binding to various types of filters that can result in product loss. However, these inert filters are inappropriate for removing cells and cell debris from suspension cultures because of their low capacity. We have selected various kinds of filters from major filter manufacturers, screened and compared the resulting filter capacities and AAV recoveries. In addition, several combinations of depth filters were investigated and a clarification process using a train of two Depth Filters in combination with a sterile filter provided the best combination of capacity and recovery. The clarification process has been scalded up to 50L suspension cell culture, no AAV product loss was observed. We believe this clarification process, which is GMP compliant, single use, higher yield, low risk, is scalable to harvesting 2000 L AAV from suspension culture.

338. Addressing Large-Scale Viral Vector Manufacturing Using an Optimized Pei-Based Transfection Process

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With the progress in developing new viral vector systems guided by safety, specificity and potency considerations, several gene and cell based therapies are now more than ever closer to being clinically approved and commercially available to treat genetic diseases. Viral vector delivery systems, of which mainly adeno-associated viruses (AAV) and lentiviruses are produced by transient transfection of mammalian producer HEK-293 cell lines. Virus vector production using the right transient transfection method is crucial to provide the flexibility and reproducibility that is needed to scale-up from initial process development to manufacturing of high-quality grade viral vectors. Here, we describe an optimized PEI-based virus production process for high-yielding viral vector production, compatible with different cell culture adherent and suspension systems. We further demonstrate the robust viral vector production yields, as well as the adaptability and reliability of the PEI-based transient gene expression approach to efficiently manufacture GMP-grade viral vectors at a sufficiently large scale for more advanced clinical trials, and *in fine* to drive commercialization of therapeutic vectors.

339. Serum Free and Chemically Defined Platform for the Growth and Propagation of HEK293 Cells and Adenovirus Viral Vector Amplification

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Current viral vector manufacturing processes face a number of significant challenges. Typical vector production schemes involve propagating seed trains and viral amplification in cultures using serum containing media. Although these serum containing media deliver robust viral vector yields, the serum component has several disadvantages such as variability of performance, high costs, and potential of introducing adventitious agents. Utilizing serum free medium for growth and amplification answers these concerns and in addition can lead to significant process improvements. HEK293 cultures adapted to serum free conditions provides improvements to scalability, significant cost savings, and reduced regulatory burden. We evaluated multiple serum free, animal component free, and chemically defined media formulations for an adenovirus vector production in the iCELLis bioreactor. These media were evaluated for ease of adaptation and growth characteristics with HEK293 cells previously adapted to serum free conditions. The adenovirus vector used in this evaluation was a replication deficient adenovirus serotype 5 (Ad5) vector containing a transgene for a synthetic fluorescent protein. Process parameters such as multiplicity of infection (MOI) and duration of infection (DOI) were optimized to maximize vector amplification.

Clinical Trials Spotlight I

340. Gene Therapy for X-Linked Chronic Granulomatous Disease

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X-linked Chronic Granulomatous Disease (XCGD) results from mutations in *CYBB* encoding the gp91phox subunit of phagocyte NADPH-oxidase. G1XCGD is a self-inactivating lentiviral vector

in which a myeloid-specific chimeric promoter drives gp91phox expression from codon-optimized cDNA. Pre-clinical studies of G1XCGD demonstrated safety and efficacy to correct neutrophil oxidase in XCGD mice and in human xenografts of transduced XCGD patient-derived CD34+ cells in NSG mice. A trial of gene therapy using G1XCGD was approved by regulatory agencies in the US and UK. All enrolled patients had history of severe, persistent infections. We report results for 6 patients (2-27 years of age) with 7- to 25-month follow-up. Two additional patients were enrolled, but died of complications from pre-existing conditions ≤2 months after treatment. A back-up G-CSF-mobilized leukopheresis product was first stored, a 2nd mobilization/collection (G-CSF & Plerixafor) was performed ≥1 month later, and selected CD34+ cells were transduced with G1XCGD. Subjects received near-myeloablative conditioning with busulfan, targeted to net AUC 65-77 mg*hr/L. Autologous CD34+ cells transduced with the G1XCGD vector were infused IV either fresh or after cryopreservation. VCN of the drug product was 0.7-5.5. Infusions were well-tolerated and there were no vector-related adverse events. Subjects experienced typical conditioning-related transient neutropenia, thrombocytopenia and mucositis. Follow-up demonstrated sustained persistence of 10-50% oxidase positive neutrophils in circulation and stable vector copy numbers in blood cells at 0.1-1.2 copies/cell. Patients have been well, without new infections, and most are off prophylactic antibiotics. These results demonstrate efficacy and favorable safety profile of this treatment for XCGD.

341. Phase I / II Clinical Trial for Recessive Dystrophic Epidermolysis Bullosa Using EB-101 (COL7A1 Gene-Corrected Autologous Keratinocytes)

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Recessive Dystrophic Epidermolysis Bullosa (RDEB) is an inherited genetic skin disorder characterized by chronic blistering, open and painful wounds, joint contractures, and shortened life span. RDEB patients lack functional type VII collagen (C7) owing to mutations in the gene *COL7A1* that encodes C7, the main component of anchoring fibrils (AF) which stabilizes the dermal-epidermal basement membrane. A Phase I/II clinical trial evaluating wound closure after EB-101 (*ex vivo* gene corrected keratinocytes) treatment was evaluated in patients with severe RDEB subjects (n=7, 42 total treatments). Autologous RDEB keratinocytes isolated from skin biopsies were transduced with retrovirus encoding full-length *COL7A1* and applied onto non-healing chronic wounds with a mean time open prior to treatment of 8.5 years. No serious adverse events have been reported and replication competent retroviruses have not been detected for up to 3 years. Wound healing defined as >50% healing compared over baseline has been observed in 100% (42/42 grafts) at 3 months, 90% (38/42) at 6 months, 63% (24/38) at 12 months, and 81% (21/26) at 24 months. By comparison, untreated control wounds healing >50% have been observed in 20% (2/10) at 3 months, 10% (1/10) at 6 months, 0% (0/8) at 12 months, and 0% (0/2) at 24 months. In addition, C7 expression was observed in biopsies of EB-101 treated wounds up to 2 years post-treatment. The ongoing Phase I/II trial results demonstrate *COL7A1 ex-vivo* gene transfer has a favorable safety profile and significant wound healing efficacy that correlates with C7 molecular correction.

Keywords: Epidermolysis Bullosa, COL7A1, gene therapy, clinical trials

Phase 1/2 EB-101 (COL7A1 gene-corrected autologous keratinocytes)

Months	3	6	12	24	36
Subjects n	7	7	6	4	1
EB-101 (Col7 gene corrected grafts)	42	42	38	26	6
Wound Healing >50% n	42	38	24	21	6
Wound Healing >50%	100%	90%	63%	81%	100%

342. A Phase I Clinical Trial of Malignant Pleural Disease Treated with Regionally Delivered Autologous Mesothelin-Targeted CAR T Cells: Safety and Efficacy - A Preliminary Report

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Background: Malignant pleural disease (MPD) from primary malignant pleural mesothelioma (MPM) or secondary metastatic disease (lung and breast cancers) affects more than 150,000 patients a year in the US alone. MPM is a regionally aggressive malignancy with a poor median survival of 9 to 17 months, even after trimodality therapy consisting of chemoradiation and aggressive surgical resection. We developed chimeric antigen receptors (CARs) to target mesothelin (MSLN), a cell-surface antigen that we have shown to be highly expressed in MPM and that is associated with invasion, MMP-9 secretion, and poor survival, with low expression in normal tissues. **Methods:** Using a second-generation CD28-costimulated MSLN CAR with the Icaspase-9 safety gene (IcasM28z), we initiated a phase I clinical trial (NCT02414269) to determine the safety and maximum tolerated dose of intrapleurally administered CAR T cells. Patients with biopsy-proven MPD expressing MSLN are eligible for the study. A single dose of IcasM28z CAR T cells was administered intrapleurally (with or without cyclophosphamide preconditioning) by either pleural catheter or an interventional radiology procedure. To monitor on-target, off-tumor toxicity, serial serum-soluble MSLN-related peptide (SMRP) and C-reactive protein levels were measured and CT and PET scans were performed, in addition to routine clinical and laboratory tests. **Results:** Twelve patients with MPD (10 MPM, 1 lung cancer, 1 breast cancer) were treated (prior lines of therapy 1-7). Nine of the 12 patients (cohorts 2 to 4—3E5, 1E6, and 3E6 CAR T cells/kg) received cyclophosphamide preconditioning; the first cohort did not receive

cyclophosphamide. Three patients were administered CAR T cells using an interventional radiology procedure. One patient had febrile neutropenia (grade 3) related to cyclophosphamide. No CAR T-cell-related toxicities higher than grade 1 were observed. The last cohort of patients were admitted 2 weeks after infusion with a temperature of 101°F and fatigue. Intense monitoring for on-target, off-tumor toxicity by clinical (chest or abdominal pain), radiological (CT/PET or echocardiogram for pericardial effusion, ascites), laboratory (troponin elevation), and other (EKG) evaluation found no evidence of toxicity. One patient successfully underwent curative-intent surgical resection 6 weeks after CAR T-cell infusion. CAR T cells were detected in the peripheral blood of 6 patients (day 1 to 31 weeks), as evidenced by vector copy number. T-cell persistence was associated with decreased serum SMRP levels (>50% compared to pretreatment) and evidence of tumor regression on imaging studies (5 patients). Once lack of toxicity had been established (6-17 weeks after CAR T-cell infusion), 7 patients received anti-PD1 checkpoint blockade agents (1-10 cycles), off protocol, with no toxicity. One patient with MPM had complete metabolic response on PET scan (3E5 CAR T cells/kg and 10 cycles of anti-PD1 therapy to date); this patient remains clinically well 8 months after CAR T-cell infusion, with evidence of CAR T-cell persistence in peripheral blood and tissue at 31 weeks. **Conclusion:** In this phase I clinical trial, intrapleurally administered MSLN-targeted CAR T cells have been well-tolerated, with no evidence of on-target, off-tumor or therapy-related toxicity and with evidence of CAR T-cell antitumor activity. MSLN-targeted CAR T-cell therapy combined with anti-PD1 agents shows encouraging clinical outcome.

343. Autologous Tgfβ-Resistant HPV-16/18 E6/E7-Specific T Lymphocytes with or without Lymphodepletion for the Treatment of HPV-Associated Cancers

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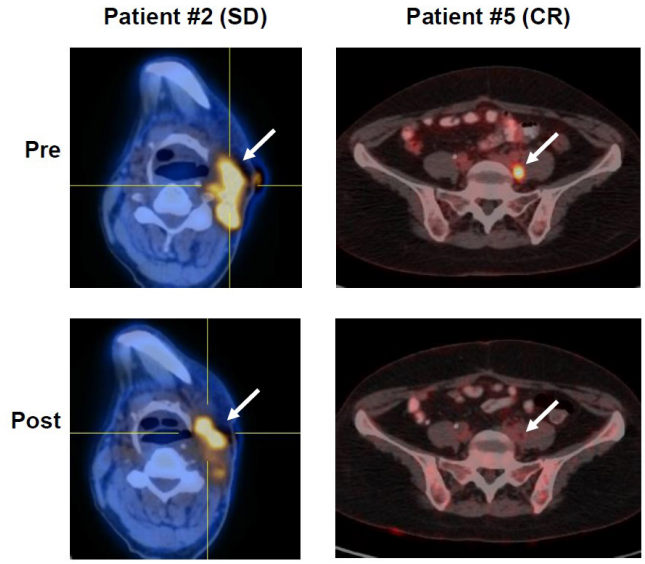
Introduction: Human papillomavirus (HPV) types 16 and 18 are strongly associated with several different cancers, all of which express the viral E6 and E7 antigens that play a key role in the deregulation of the cell cycle and oncogenesis. These oncoproteins are thus attractive therapeutic targets for immunotherapy in patients with HPV-associated cancers. These tumors often also produce transforming growth factor-β (TGFβ), which markedly inhibits antigen-specific T-cell immunity. EBV-specific T lymphocytes transduced with a dominant negative TGFβ receptor have demonstrated significant antitumor activity (Bollard et al., J Clin Oncol. 2018). We therefore developed a phase 1 dose-escalation clinical trial (NCT02379520) for patients with relapsed HPV-associated oropharyngeal and anogenital malignancies to study the safety and efficacy of autologous HPV-specific T cells (HPVST) transduced with a dominant negative receptor for TGFβ (TGFβ-DNRII). **Methods:** Eligible patients are enrolled into 2 cohorts: cohort A patients receive HPVSTs dose level 1 (1×10^7 cells/m^2) or 2 (3×10^7 cells/m^2) without prior lymphodepletion; after confirmation that dose level 2 is safe, cohort B patients receive HPVSTs at dose level 2 or 3 (1×10^8 cells/m^2) in combination with both preceding lymphodepletion (cyclophosphamide 500 mg/m^2/day and fludarabine 30 mg/m^2/day × 3 days) and nivolumab 240 mg every 2 weeks. Toxicity is evaluated using NCI Common Toxicity Criteria Scale Version 4. Response is evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) criteria. Quantitative real-time PCR is used to detect the presence of the transgene in circulation post HPVST infusion. **Results:** Six patients have been treated so far. Three males and 3 females, with a median age of 60 years (range 34 to 66) received a median of 2 infusions (1 to 3) of HPVSTs at dose level 1 for 2 patients and at dose level 2 for 4 patients. Four patients had oropharyngeal and 2 patients had cervical cancer. There have been no treatment-related dose-limiting toxicities (DLTs) and no grade 3-5 immune-related adverse events. In cohort A, after one infusion of HPVSTs, 3 patients had transient stabilization of disease (illustrated for patient #2 in the figure below) and 1 developed progressive disease; in cohort B, 1 patient achieved complete response (patient #5) and 1 had progressive disease. Quantitative real-time PCR for TGFβ-DNRII in peripheral blood shows peak expansion of the infused HPVSTs 1 to 2 weeks after infusion, with detection up to 3 months. The mean transgene copy number at 1 week in cohort A and B was 124 (±121) and 21,994 (±9,956) per mg of genomic DNA, respectively. **Conclusions:** Preliminary data from this phase 1 dose-escalation study suggest that TGFβ-DNRII-modified HPVSTs have a manageable safety profile in patients with relapsed HPV-associated malignancies. Administration after lymphodepleting chemotherapy is associated with ~180-fold greater expansion of HPVSTs in vivo. Promising signs of clinical efficacy warrant further study.



344. Treatment of Recurrent HGG Patients with the Retroviral Replicating Vector Toca 511 and Toca FC Gives Durable Responses and Survival Lasting 3 Years or Longer: Immune Mechanisms and Molecular Analyses of Tumors

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Toca 511 is a retroviral replicating vector (RRV) based on an amphotropic gammaretrovirus that encodes an optimized humanized yeast cytosine deaminase (CD) and Toca FC is an extended release formulation of 5-Flucytosine (5-FC) which is converted to 5-Fluorouracil (5-FU) by CD, but not by any human gene activity. Toca 511 shows preferential uptake by tumor cells leading to local chemotherapy without systemic activity or toxicity. We have shown in animals that this treatment leads to high 5-FU levels in infected tumors, disruption of the immune suppressive tumor microenvironment, depletion of myeloid immune suppressive cells and robust anti-tumor CD4 and CD8 T cell responses. A phase 1 dose escalation trial (NCT01470794) with Toca 511 and Toca FC in patients with recurrent High Grade Glioma who underwent resection and injection of Toca 511 into the resection bed followed by cyclic treatment with Toca FC has completed enrollment and we continue to follow patient outcomes. Typically these tumors recur rapidly and median survival of such patients is around 8-9 months. The treatments were well-tolerated with no dose-limiting toxicities observed. Among 53 patients that were efficacy evaluable we report on the long term outcomes for 23 patients in the highest dose group where more than 25% of patients continue to survive (the two longest are currently over 50 months) and 5 of the long term survivors have durable ongoing complete responses lasting longer than 36 months. The late-occurring durable clinical responses suggest that these responses have a strong immune component, as seen in animal models. We now report on the initial analyses of the immune status of tumors before treatment, including neo-antigen load, in responding versus non-responding patients, and on the search for changes in systemic markers post treatment We also report on the molecular analyses of the virus recovered from treated patients in this trial and after iv administration of Toca 511 (NCT01985256), and preclinical data showing that efficacy can be observed with low levels of tumor transduction. These data support the ongoing Phase 3 "Toca 5" trial (NCT02414165) in recurrent high grade glioma patients.

345. Phase I/II Trial Oof Intratumoral and Resection Cavity Administration of an Edmonston Oncolytic Measles Virus (MV) Derivative Expressing the Human Carcinoembryonic Antigen (CEA) in Patients with Recurrent Glioblastoma

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MV vaccine strains have significant antitumor activity against GBM, the most common and lethal glioma histology (Phuong et al, Cancer Res, 2003). Oncolytic selectivity is in part due to MV receptor CD46 overexpression in GBM cells. We hypothesized that MV will be safe and effective in GBM treatment, and that engineering MV to express the marker peptide CEA (MV-CEA) could allow real-time monitoring of viral gene expression; we also sought to identify tumor characteristics that predict treatment efficacy. Eligible patients (pts) had recurrent GBM (rGBM), protective anti-MV antibody titers and normal CEA levels. MV-CEA was administered either at the resection cavity (arm A), or, intratumorally on day 1, followed by a second dose administered in the resection cavity after tumor resection on day 5 (arm B). The standard cohorts of three design was applied. Nine evaluable pts were enrolled in arm A and 13 evaluable pts were enrolled in arm B with ten of those pts receiving treatment at the maximum dose level. Results: No dose-limiting toxicity was encountered; doses 10^5 - 10^7 TCID50 were administered in arm A and $2x10^6$ - $2x10^7$ TCID50 were administered in arm B: $2x10^7$ TCID50 was determined to be the phase II dose (maximum feasible dose). Treatment-related toxicities were minimal: for both treatment arms, combined grade 2 toxicities were limited to 2 pts with grade 2 fatigue, 1 pt with grade 2 anemia, and 1 pt with grade 2 lymphopenia . Median progression free survival was 3.8 mo and median overall survival was 11.8 mo, which compares favorably with contemporary controls; 89% of pts in arm A and 92.3% pts in arm B accomplished disease stabilization. There was no treatment-induced immunosuppression as assessed by DTH, CD4, CD8 and complement levels, and no shedding in urine or mouth gargle specimens. Immunohistochemistry for measles virus N protein and QRT-pCR to assess viral replication was performed both at baseline, and at the time of tumor resection of paired MV treated tumors. Variable levels of virus replication up to 6×10^7 genome copies/μg RNA was observed in 9/10 tested tumors. A custom made 790 NanoString gene panel was employed in parallel to assess gene expression: a diagonal linear discriminate analysis (DLDA) classification algorithm, we generated in patient derived GBM xenografts, was applied and values in baseline samples were found to be strongly ($p=0.03$) and inversely ($p=-0.717$) correlated with viral replication. The 22 interferon stimulated gene (ISG) based DLDA algorithm was able to accurately distinguish tumors into highly permissive, moderately permissive and resistant to MV replication. The MV receptor CD46 was expressed in all tumors and did not impact permissiveness. In summary, MV-CEA has excellent safety and promising clinical and biologic activity in treatment of rGBM.

Viral replication is reversely associated with ISG expression in tumors: the predictive DLDA algorithm we developed represents a useful tool for patient selection and treatment customization, and it is currently prospectively validated. Supported in part by NIH R01CA154348, P50CA108961 and R01CA200507

Hematologic & Immunologic Diseases III, Cancer-Targeted Gene & Cell Therapy II

346. Towards Breaking Immune Tolerance of Tumors by Transplantation of Hematopoietic Stem and Progenitor Cells Engineered to Express Interferons in Their Tumor-Infiltrating Myeloid Progeny

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While chemotherapy still represents the most used therapy for disseminated malignancies, most of the times it allows symptom relief, delaying disease progression rather than providing a definitive cure. Much efforts are being put into the development of immunotherapies capable of eradicating the disease, but important challenges include the lack of strong tumor cell-specific antigens and the requirement to overcome the immuno-suppressive tumor microenvironment (TME) often subverting the efficacy of immunotherapies. Employing bone marrow transplantation of hematopoietic stem and progenitor cells (HSPCs) genetically modified with a transcriptionally- and post-transcriptionally regulated lentiviral vector cassette, we have previously shown that selective delivery of type 1 interferons (IFNa) into the TME by tumor-infiltrating myeloid cells can render such microenvironment permissive to the induction and execution of an antitumor immune response in solid tumor models and, more recently, in an aggressive model of B-acute lymphoblastic leukemia (B-ALL). Briefly, IFNa gene therapy enhanced the spontaneous generation of T cells with reactivity to multiple tumor associated antigens, promoted epitope spreading from strong to weaker antigens and augmented anti-tumor activity of adoptively transferred T cells expressing transgenic TCRs or CARs (Escobar et al, under revision). Using the same delivery approach, we have now explored type 2 interferon (IFNg) as an alternative payload that may induce anti-tumor immunity through additional and potentially complementary mechanisms. Transplantation of HSPC transduced with a lentiviral vector expressing IFNg under transcriptional control of the Tie2 promoter (to target expression to tumor-infiltrating myeloid cells) and post-transcriptional control by miR-126/miR-130a (to prevent expression in HSPC) was well tolerated by the mice, which showed normal blood counts and no obvious signs of toxicity. To test the anti-tumor efficacy of IFNg, we employed 2 models. As a first model, we investigated a colorectal carcinoma model inoculated subcutaneously in animals with previously transplanted IFNg-engineered or control HSPCs. Compared to control animals, IFNg gene therapy significantly reduced tumor growth, which was accompanied by an increase of CD8+ T cells, a decrease of myeloid-derived suppressor cells as well as a reduction in vascularization within the TME. Notably, we observed a significant reduction of tumor growth in a spontaneous murine B-ALL model. IFNg gene therapy inhibited leukemia growth to a similar extent as IFNa and led to upregulation of MHC-II expression on dendritic cells and macrophages within the TME indicating reprogramming of the tumor immune infiltrate to a more immune-responsive state. Mechanistic studies on differential activities of IFNg vs. IFNa, as well as combination therapies including check-point blockers, are under way. In conclusion, HSPC-based delivery of genetically encoded anti-tumor cytokines holds great promise as a novel immunotherapeutic strategy to break immune tolerance of tumors.

347. Gene Therapy for Chronic Eosinophilic Leukemia

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Eosinophils are bone marrow-derived granulocytic cells that play a role in combating parasites and other pathogens. In normal individuals, eosinophils represent <5% of peripheral leukocytes (300-500/μl), persist in the circulation 8 to 12 hr, survive in tissues 8 to 12 days, and carry a variety of cytotoxic mediators in cytoplasmic granules. If eosinophils invade tissues in sufficient numbers, they can cause significant organ damage and dysfunction. There are a variety of hypereosinophilic disorders characterized by chronic elevation of blood eosinophil levels, invasion of organs with eosinophils, and associated organ damage. Chronic eosinophilic leukemia-not otherwise specified (CEL-NOS), a subtype adult chronic eosinophilic leukemia characterized by persistent elevation of blood eosinophils ($>1.5x10^3$/μl) of unknown cause, is characterized by dysfunction of organs infiltrated with eosinophils and is unresponsive to any therapy. The disease is characterized clinically by weight loss, cough, weakness, diarrhea, splenomegaly, hepatomegaly and cardiac and lung dysfunction. Since the pathogenesis of this disease is unknown, the most direct therapy is to suppress the number of eosinophils in blood, thus suppressing eosinophil tissue invasion and organ dysfunction. Based on this concept, we hypothesized that a one-time gene therapy for CEL-NOS using an adeno-associated virus (AAV) vector coding for an anti-eosinophil monoclonal antibody (AAVrh.10mAnti-Eos) will provide sustained expression of the transgene, while lowering blood and tissue eosinophil levels *in vivo*. AAVrh.10mAnti-Eos codes for an anti-eosinophil [Siglec-F (mouse)] monoclonal antibody that induces eosinophil apoptosis. To evaluate the effectiveness of AAVrh.10mAnti-Eos, a CEL-NOS mouse model was created using a vector expressing the cytokine IL-5 (AAVrh.10mIL-5), which induces bone marrow differentiation of eosinophils. To evaluate apoptosis of eosinophils, white blood cells were stained with anti-CCR3 (to identify eosinophils), incubated with lactadherin, (to identify cells in the early stages of apoptosis) and analyzed by flow cytometry. AAVrh.10mIL-5 administered intravenously to wild type mice stimulated the bone marrow to persistently generate high blood levels of eosinophils ($>10^5$ eosinophils/μl), with tissue eosinophil infiltration in heart, lungs and liver and eventually death. AAVrh.10mAnti-Eos (2.5×10^{10}) induced

apoptosis of circulating eosinophils *in vivo*, markedly lowered blood eosinophil levels in the CEL-NOS mouse model (p=0.02) and reduced mortality (p=0.07) when compared with untreated mice. These results suggest that a single treatment with AAVrh.10mAnti-Eos has the potential to provide substantial therapeutic benefit to patients with CEL-NOS, a fatal malignant disorder, to protect from chronic infiltration of eosinophils and improve quality of life and could be applied to other eosinophilic disorders that have unmet treatment needs.

348. Toward a Hematopoietic Stem Cell-Based Prophylactic Immuno-Gene Therapy Approach for Cancer

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Our ultimate goal is to develop a long-lasting, cost-efficient, and technically simple approach that allows for the immuno-prophylaxis of cancer in patients with high-risk for disease recurrence and, ultimately, in patients with cancer-predisposing inherited mutations. Our approach is based on *in vivo* genetic modification of hematopoietic stem cells (HSCs). Because HSCs are self-renewing and give rise to all blood cell lineages, they provide a life-long source of transgene modified myeloid and lymphoid cells that, during tumor development, infiltrate the tumor and support tumor growth. Our in vivo HSC transduction approach involves subcutaneous injections of G-CSF/AMD3100 to mobilize HSCs from the bone marrow into the peripheral blood stream and the intravenous injection of an integrating, helper-dependent adenovirus (HD-Ad5/35++) vector system. In preliminary studies we used an integrating HDAd5/35++ vector expressing GFP and mgtm^{P140K} under the ubiquitously active EF1α promoter. We demonstrated in adequate mouse models that HSCs transduced in the periphery home back to the bone marrow where they persist long-term. Transgene expression in peripheral blood cells can be achieved by O6BG/BCNU injection. Short-term exposure of *in vivo* transduced mice to low 6BG/BCNU doses resulted in stable GFP expression in 80% of peripheral blood cells and, in mice with implanted tumors, in 80% of tumor infiltrating leukocytes. The predominant GFP+ cell type in the tumor was tumor-associated neutrophils (Ly6G$^+$) followed by macrophages (F4/80$^+$/MHCII$^+$). To avoid adverse reactions using our *in vivo* HSC transduction/selection approach, the expression of therapeutic transgenes has to *i)* be localized to the tumor, *ii)* be automatically activated only when the tumor begins to develop, and *iii)* cease when the tumor disappears. To develop such an expression system, we determined (by miRNA-Seq and miRNA-array) the micro-RNA profile in GFP$^+$ cells isolated from the bone marrow HSCs, spleen, PBMCs, and tumors of in vivo transduced mice. We found four miRNAs that were expressed at high levels in HSCs, splenocytes and PBMCs, but were absent in tumor-associated leukocytes. By inserting four copies of the corresponding target sites into the 3'UTR of our GFP transgene, the corresponding mRNA is degraded in all cells except tumor-associated leukocytes allowing for tumor-restricted transgene expression. Ongoing work is focused on miRNA-regulated vectors expressing immune checkpoint inhibitors, e.g. anti-CTLA4-scFv. We expect that after in vivo HSC transduction with this vector, mice will control the development of implanted or spontaneous tumors.

349. Lentiviral Gene Therapy for Severe Wiskott-Aldrich Syndrome - Longer Term Follow Up London Experience in 6 Patients

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Wiskott-Aldrich syndrome (WAS) is a rare primary immunodeficiency characterised by thrombocytopenia, eczema, susceptibility to infection and high incidence of autoimmunity and malignancy. Hematopoietic stem cell transplantation (HSCT) is usually curative but in the context of non-family matched donors can be associated with significant complications. Here we report a follow up to the UK experience using a SIN-lentiviral vector (LV _1.6WASp) to transduce autologous HSCs in 6 patients (NCT01347242 and NCT02333760). Patients were aged between 6 months and 30 years of age (median 8.5 yrs) and had clinically severe disease. Two patients were splenectomised prior to treatment. Length of follow up is between 3-83 months (median 26 months) and all patients are alive. Subjects received a reduced intensity conditioning regime prior to infusion of gene corrected CD34+ cells. In the first two patients bone marrow was harvested whilst GCSF/Plerixafor mobilised HSCs were harvested by leukapharesis in the remainder. Four patients experienced inflammatory symptoms related to GCSF administration. Infused cell dose ranged from 2-16.6 x 10^6 CD34+/kg with a vector copy number of 0.71-2.7 copies/cell. 5 patients received a fresh cell product and one patient, treated off-trial, received both fresh and cryopreserved transduced cells. In the 4 patients with over 12 months follow up there have been no severe bleeding episodes, frequency of infections has reduced allowing partial withdrawal from immunoglobulin and antibiotic prophylaxis, and there has been a very significant improvement in inflammatory complications including eczema. In two patients who were not previously splenectomised, platelet counts did not increase after gene therapy, and one elected for splenectomy for quality of life reasons with immediate normalisation of platelet numbers 3 years after gene therapy. In the youngest patient (treated at 11 months of age) platelet count normalised after gene therapy alone. Vector copy number in granulocytes was 0.03-1.74 copies/cell. There was selective accumulation of transduced cells in all lymphocytic lineages, most pronounced in T cells. Recurrence of autoimmunity/autoinflammation has been observed in 3 patients. One patient with high level gene marking in all lineages developed *de novo* nephrotic syndrome 2 years after gene therapy. Another who had previously rejected an allograft was difficult to mobilise and engrafted slowly requiring infusion of back-up stored cells, probably as a result of previous marrow exposure to alkylating agent conditioning. There have been no vector related adverse events, and integration profiles are highly polyclonal. Clinical improvement and gene marking has been sustained in all patients with >12 months follow up suggesting that autologous gene therapy is a rational alternative to HSCT where matched family donors are unavailable even though, as with allogeneic procedures, some disease manifestations may recur or persist.

350. SPK-8011: Preliminary Results from a Phase 1/2 Trial of Investigational Gene Therapy for Hemophilia Confirm Transgene Derived Increases in FVIII Activity That Are Persistent and Stable beyond Eight Months

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Background: Hemophilia A therapy is based on intravenous administration of exogenous FVIII protein either to treat or prevent bleeding. Prophylaxis has revolutionized health outcomes in hemophilia by significantly reducing the frequency of bleeding, preventing the development of arthropathy, improving health-related quality of life and enabling affected individuals to increase participation in physical activities enhancing psychosocial outcomes. However, the burden of repetitive infusions may be partially responsible for a strikingly high rate of non-compliance. A multi-center study revealed only 43% of individuals with hemophilia adhered to their prophylactic regimen (Schrijvers 2016) putting improved outcomes in jeopardy. Recombinant adeno-associated viral (rAAV) vectors have been in development for >25 years. No major safety concerns have emerged in the >100 previously conducted rAAV gene transfer clinical trials. A single administration of rAAV vector encoding human coagulation *F8* or *F9* gene may result in sustained expression of therapeutic factor activity levels sufficient to reduce or eliminate the need for exogenous factor infusions (Pasi 2017; George 2017). **Objectives:** 1) To safely obtain consistent, predictable and sustained FVIII activity (FVIII:C) >12% adequate to prevent spontaneous bleeding without the need for prophylactic FVIII infusions, manipulation of normal coagulant or anticoagulant pathways or increased thrombotic risk; 2) Minimize a dose-dependent capsid immune response by using the lowest possible vector dose that produces clinically relevant improvements in FVIII:C. **Methods**: SPK-8011 is a rAAV vector composed of a hepatotropic bio-engineered capsid (AAV-Spark200) and a codon-optimized expression cassette encoding a B-domain-deleted (BDD) human FVIII gene. The phase 1/2 trial is an open-label, non-randomized, dose-escalation study of SPK-8011 (starting dose $5x10^{11}$ vg/kg) in adult HA males (FVIII:C ≤2%). Laboratory data, bleeding frequency, and FVIII use before vector infusion are compared to post infusion values. **Results**: As of 12/7/2018, we infused 7 subjects: 2 at 5 $x10^{11}$ vg/kg, 3 at $1x10^{12}$ vg/kg, and 2 at $2x10^{12}$ vg/kg. Subjects were 28-52 years old with Spark200 Neutralizing Antibody (NAb) titers of <1:5. Soon after vector administration, all subjects discontinued FVIII prophylaxis. Increased hepatic transaminases outside the normal range have not been observed. We report here results from the first four subjects who have achieved steady state FVIII activity levels. All had significant arthropathy with 3 of the 4 having ≥ 4 target joints. Three had previous HCV exposure and one was HIV positive. Steady-state FVIII levels are 10 and 13% for the two patients infused with 5 $x10^{11}$ vg/kg, and 8 and 12% for the two subjects infused with $1x10^{12}$ vg/kg. After 1082 cumulative days of follow up in all infused patients, there have been no vector or procedural related serious **Conclusion**: Preliminary results in this phase 1/2 study demonstrate FVIII:C levels sufficient to prevent spontaneous bleeding without exogenous factor use. Therapeutic transgene-derived FVIII:C was achieved at the lowest vector dose thus far reported in HA trials. As of this report, no safety concerns have been observed.

351. Human Mesenchymal Stem Cells Genetically Engineered to Express Alpha-1 Anti-Trypsin (apceth-201) Confer a Long-Term Survival Benefit in a Lethal, Haplo-Identical Mouse Model of Graft-Vs-Host-Disease

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Acute Graft-vs-Host Disease (aGvHD) is a frequent complication associated with allogeneic bone marrow transplantation (BMT). aGvHD develops within the first 100 days after BMT. It is caused by activation of donor T cells within the graft and results in destruction of host tissues. The major target organs are the gastro-intestinal tract, liver, bone marrow, skin, and lungs. Immunosuppressants are used to manage aGvHD. However, steroid-refractory aGvHD (SR-aGvHD) develops in many cases and has an extremely poor prognosis. Therefore, new therapeuties are needed. Mesenchymal stromal cells (MSC) have been shown in clinical trials to be an effective treatment in SR-aGvHD. Unfortunately, a large proportion of the patients does not respond to this treatment. Apceth has developed a mesenchymal stromal cell-based gene therapy product termed "apceth-201". This product consists of human allogeneic MSC, engineered by lentiviral transduction to express the proteinase inhibitor, alpha-1 antitrypsin (AAT), to further augment the anti-inflammatory potential of the MSC. The protein AAT has been shown to have anti-inflammatory properties in GvHD clinical trials. Using *in vitro* assays, we showed apceth-201 efficiently suppresses T cell proliferation, as well as the release of the pro-inflammatory cytokines, TNFα, IL6, and IL8, from LPS-stimulated PBMCs. NK cell activity was also reduced when these cells were co-cultured with apceth-201 MSCs. To assess the *in vivo* efficacy of apceth-201, the human cell product was tested in two different aggressive mouse models for aGvHD. First, the product was used in a humanized mouse model of GvHD. Immunodeficient mice were sub-lethally irradiated and infused with human PBMCs. Mice were treated with apceth-201 on days +14 and +18 post-PBMC transfer. Vehicle-treated control animals succumbed quickly to GvHD (median survival: 18 days). Treating mice with native MSCs increased survival (median survival: 22 days). Survival was even further enhanced by administering apceth-201 (median survival: 36 days). Animals treated with apceth-201 showed significantly improved clinical scores and reduced levels of inflammatory markers such as IFNγ and soluble TNFα-receptors. Furthermore, apceth-201 preserved bone marrow cellularity in treated animals, indicating that the immunological attack by the human PBMCs was reduced. Next, the product was tested in a

GvHD model system which mimics closely haploidentical BMT, now being evaluated for use in the clinic. Immunocompetent animals were sub-lethally irradiated and infused with haplo-identical splenocytes to induce GvHD. On day +12 and +16 post-splenocyte infusion, mice were treated with the human apceth-201 product. Vehicle-treated control animals succumbed quickly to GvHD (median survival: 18.5 days). Treating animals with apceth-201 resulted in long term survival of 57% of the animals (greater than 60 days and ongoing). Initially, all treated animals showed clinical scores comparable to the control animals. Within a period of 25 days after the second injection, the clinical scores had returned to base line, indicating complete resolution of GvHD. Furthermore, apceth-201 preserved bone marrow cellularity in treated animals. Taken, together we demonstrate that apceth-201 shows broad-range anti-inflammatory activity and is superior to native MSCs in attenuating disease in two murine models of GvHD. These significant and long-lasting therapeutic effects warrant a rapid clinical translation of this treatment approach and have led to planning of a phase I/II study using apceth-201.

Musculo-Skeletal Diseases II

352. Progressive Myopathy in a New Mouse Model of Facioscapulohumeral Muscular Dystrophy (FSHD) Facilitates Development of Targeted Molecular Therapies

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Autosomal dominant Facioscapulohumeral muscular dystrophy (FSHD) is among the most prevalent muscular dystrophies, estimated to affect 1 in 8,333-20,000 individuals worldwide. FSHD molecular pathogenesis has been historically enigmatic, but the picture has clarified during the last decade. FSHD is now linked to aberrant expression of the *DUX4* gene, which encodes a myotoxic transcription factor. Thus, *DUX4* inhibition may be a direct path to FSHD therapy. The emergence of DUX4 as a primary pathogenic insult in FSHD represented a momentum shift in the field, as it enabled translational research for the first time. As animal models are essential tools for translational research, several labs have made important efforts to develop *DUX4*-expressing animals during the last decade. Since DUX4 is extremely toxic, animal model development has been difficult, but progress has been made, and these current systems revealed that tight regulation of DUX4 expression is critical for creating a viable model that develops in Mendelian ratios and recapitulates muscular dystrophy. Since 2009, we have been working to generate an inducible FSHD mouse model with tight regulation of *DUX4*. Here we report such a model - the TIC-DUX4 line - which utilizes Tamoxifen (TAM)-Inducible CRE recombinase to turn on DUX4 in skeletal muscle. Uninduced TIC-DUX4 (i.e. DUX4-off) mice are born in Mendelian ratios, show no DUX4 leakiness, develop normally to adulthood, and are indistinguishable from wild-type animals. Importantly, TAM-induced animals display TAM- and DUX4-dose-dependent molecular, histopathological, and functional deficits associated with muscular dystrophy. Specifically, induced mice have significantly reduced skeletal muscle force production, impaired open field activity, muscle wasting, and histological indicators of muscular dystrophy, including increased central nuclei and inflammation. Importantly, these phenotypes are tunable; myopathy progresses slowly over many months at low doses of TAM, while high doses can be used to rapidly induce widespread myopathic phenotypes within 2 weeks. Vehicle-treated TIC-DUX4 and wild-type controls show no deficits. As expected, DUX4 protein and mRNA are detectable only in skeletal muscle and correlate with increased TAM dose. We are now using this model to test DUX4-targeting therapeutic strategies, including RNAi-based gene therapy, which we report here. Specifically, we found AAV delivery of a U6-promoter driven, DUX4-targeted microRNA provided long-term protection from DUX4-associated damage in old TIC-DUX4 mice. These data will support translation of RNAi-based gene therapy for FSHD, and the TIC-DUX4 mouse model will be useful for testing other DUX4-targeted therapies as they emerge. This study was supported by the Muscular Dystrophy Association (MDA) and the National Institutes of Health, NINDS R21/R33 IGNITE grant 1R21NS101166.

353. Pre-Clinical Data and a Planned Phase One Human Trial of AAV.IL-1Ra-Mediated Gene Therapy for Osteoarthritis

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Osteoarthritis (OA) is common, debilitating, incurable and difficult to treat; it is the most common cause of disability in the US. Local, intra-articular therapy is the preferred strategy for treating OA, but it is very difficult to target drugs to joints. Gene delivery has the potential overcome this limitation and enable the endogenous synthesis of anti-arthritic gene products at therapeutic concentrations over an extended period. In this context, we have developed a recombinant AAV vector encoding the interleukin-1 receptor antagonist (AAV.IL-1Ra) for local administration into the joints as a potential therapy for OA. Preclinical pharmacology and GLP toxicology/ biodistribution studies in rats have confirmed the safety of this vector and also demonstrated persistence of vector genomes in the injected knee for at least 1 year, with minimal spread to other anatomical locations. Additional studies in horses confirmed efficient transduction of synovial lining cells and articular chondrocytes after intra-articular injection, with enhanced transgene expression in diseased joints. As in the rat studies, there was no substantial spread evidence of vector genomes in tissues other than the injected joint, and transgene expression persisted for at least one year. Collectively, the pre-clinical data suggest that AAV.IL-1Ra is effective in reducing the signs and symptoms of OA, including short-term and long-term pain, as well as in slowing the rate of degenerative changes. Given these data, AAV.IL-1Ra shows promise both to reduce pain and act as a DMOAD (disease-modifying anti-osteoarthritis drug); this

would represent a significant advance for patients since there are no currently approved drugs that can slow disease progression. A phase I clinical trial is planned to study AAV.IL-1Ra in 9 subjects with knee OA (ClinicalTrials.gov Identifier NCT02790723). This is a dose-escalating trial, with 3 cohorts of 3 patients receiving 10^{11}, 10^{12}, or 10^{13} vg per knee. Each patient will be followed for 1 year, with safety as the primary outcome measure. Acknowledgement Funded by a clinical trial grant from the Dept. Defense, number W81XWH-16-1-0540

354. BB-301: A Single "Silence and Replace" AAV-Based Vector for the Treatment of Oculopharyngeal Muscular Dystrophy (OPMD)

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BACKGROUND: OPMD is an autosomal dominant disorder that impacts the muscles of the eyelids and pharynx, leading to ptosis and dysphagia respectively, as well as can lead to proximal limb weakness. Despite the weakness associated with the other muscle groups, it is the complications of dysphagia that most often require serious intervention. The disease is caused by an abnormal expansion of alanine-encoding trinucleotide repeats in the coding region of the poly(A) binding protein nuclear-1 (PABPN1) gene. The A17 mouse model, expressing a bovine PABPN1 with an expanded polyalanine tract, recapitulates most of the features of human OPMD patients including a progressive atrophy and muscle weakness associated with nuclear aggregates of insoluble mutant PABPN1. Previous preclinical studies in A17 mice tested a two-AAV vector system for the treatment of OPMD: one vector produces short hairpin RNA (shRNAs) to silence endogenous (including mutant) PABPN1. The second vector expresses a codon-optimized version of wildtype PABPN1 that takes advantage of amino acid codon degeneracy to produce a wildtype protein that is not cleaved at the RNA level by the anti-PABPN1 shRNAs. Co-administration of both vectors into tibialis anterior (TA) muscles resulted in improvement of many of the disease phenotypes including restoration of muscle strength to wildtype levels. RESULTS: Here we describe the development of BB-301, a single vector "silence and replace" therapeutic comprised of an AAV9 capsid to deliver a recombinant genome that uses a single muscle specific promoter to produce a bifunctional RNA that expresses shRNA against PABPN1 as well as a codon-optimized shRNA-insensitive wildtype PABPN1. By taking advantage of the existing endogenous RNAi machinery, and the small size of the sequences encoding the shRNAs, leaves sufficient packaging capacity for the co-expression of modestly sized genes resulting in a single vector with 'silence and replace' capabilities. In a 20-week experiment, treatment of TA muscles with BB-301 at a dose of 6e10 vg/muscle results in robust inhibition of mutant PABPN1 expression by up to 87% and restores wildtype PABPN1 levels up to 91% of endogenous levels. Concomitantly, BB-301 treatment resulted in correction to near wildtype levels of intranuclear inclusions, fibrosis, and muscle strength as assessed by maximal force. A follow-on dose ranging experiment was performed over 14-weeks at administered levels from 4e8 vg/muscle to 7.5e11 vg/muscle. Mid-ranged doses of BB-301 that result in 75% inhibition of mutant PABPN1 and 26% restoration of wildtype PABPN1 produces full phenotypic correction of muscle strength and muscle weight, suggesting that BB-301 may provide a broad therapeutic window. CONCLUSIONS: Cumulatively, these data support the use of a single vector "silence and replace" based approach to treat OPMD. A first-in-man study, in which BB-301 will be injected directly into the cricopharyngeus muscle for treatment of OPMD-related dysphagia, is anticipated in Q4 2018.

355. Identification of Novel AAV Capsids for Skeletal Muscle Gene Transfer by *In Vivo* Selection in Humanized Mice

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Recombinant adeno-associated viral (AAV) vectors have shown efficacy and safety in numerous gene therapy applications in animal models and human clinical trials. Development of AAV gene therapies has relied on a small number of capsids identified from natural sources that proved to be considerably more efficient than AAV2 for *in vivo* gene transfer while retaining similar safety profiles. However, the development of novel capsids provides the opportunity to reduce manufacturing requirements and broaden the treatment population to include patients who are seropositive for traditional AAVs. Numerous approaches have been employed recently to develop novel capsids with unique properties, such as highly efficient transduction of specific tissue/cell types and low prevalence of neutralizing antibodies. Here we describe two novel AAV capsids with tropism for skeletal muscle and their therapeutic potential for muscular dystrophies. In particular, Duchenne muscular dystrophy (DMD) is an X-linked recessive genetic disease that affects 1 out of every 3,500 to 5,000 male births, has no current treatment, and an average life expectancy of about 30-40 years with palliative care. Identifying AAV capsids with higher muscle gene transfer efficiency after systemic administration will expand the range of options for patients, reduce the risk of adverse responses, and improve the treatment economics. A DNA shuffled AAV capsid library with ~$2x10^7$ unique members was injected IV via tail vein into *NOD-Rag1^{null}IL2ry^{null}* immunodeficient mice with an engrafted human donor muscle in one hindlimb. Three doses were injected and mice were sacrificed 4 weeks post-injection. Genomic DNA was isolated from the human xenograft in each dose group, and AAV capsids were PCR amplified from these samples and Sanger sequenced. After one selection round, approximately 60 capsid sequences were initially

identified, representing a wide range of parental capsid combinations. Recombinant AAV vectors encoding GFP were produced and tested first in cell culture. A number of vectors were selected for *in vivo* testing based on phylogenetic diversity, production efficiency and gene transfer efficiency in cell culture. *In vivo* testing was conducted in C57BL/6J and *DMD*^{mdx} mice at ~5E13 vg/kg for biodistribution and GFP expression. Two novel capsids, AAV-X3_9 and AAV-X4_10, were identified to transduce muscle and drive GFP expression levels similar to AAV9 in both C57BL/6J and *DMD*^{mdx} mice. Of particular interest, AAV-X3_9 showed lower transduction of liver, spleen and lung than AAV9. We compared the human serum neutralization profile of both AAV-X3_9 and AAV-X4_10 to AAV1, AAV8 (their closest related parental capsids, respectively) and AAV9, and both new capsids show less neutralization than AAV1 or AAV9 using serum from 12 random, healthy adult humans. In conclusion, we describe two new capsids with similar muscle transduction and gene expression as AAV9. In addition to the current results, these capsids are new platforms that will be used to perform additional optimization to achieve higher muscle gene transfer with reduced NAB profile than existing capsids.

356. AAV Therapy Attenuates Respiratory Dysfunction in a Novel Rat Model of Pompe Disease

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Introduction. Pompe disease is defined by a deficiency or absence of acid-alpha glucosidase (GAA) and characterized by the systemic depletion of GAA resulting in ubiquitous lysosomal glycogen accumulation. Respiratory dysfunction is prominent in patients with Pompe yet the mechanism defining the development of muscle weakness is currently unclear. The transgenic knockout mouse model of Pompe disease has been invaluable in mechanistic and therapeutic studies but is limited in several key areas. Here, we describe a novel rat knockout model of Pompe disease created by zinc finger nuclease (ZFN) technology. The ZFNs demonstrate high specificity to the targeted region of *Gaa* resulting in complete absence of the enzyme. *Gaa* deficiency results in an accelerated accumulation of lysosomal glycogen and pathology in the rat knockout model which is more analogous to clinical presentation and serves as an alternative model for evaluation of therapeutic interventions. Here we examined respiratory function following intravenous administration of AAV-GAA in the rat model of Pompe disease. Methods. Zinc finger nucleases were designed to disrupt the rat Gaa gene, resulting in global knockout of Gaa (KO). Male Sprague-Dawley rats were divided into the following groups: wild-type (WT), KO, and KO+AAV9-hGAA (KO+AAV). KO+AAV animals received a single intravenous administration of AAV9-hGAA at post-natal day 0. Animals were monitored daily and respiratory function was assessed via plethysmography at 5 months of age. At study end-point (5 months) biodistribution of transgene expression in respiratory muscles and spinal cord was determined. GAA activity in the diaphragm and glycogen deposition were assessed in each cohort. Results. All males (5/5) that received AAV survived to 5.5 months, whereas KO rats had a median survival of 5 months and ~100% mortality rate prior to 6 months of age. Reduction of glycogen deposition and elevation of GAA activity was observed in KO+AAV diaphragm lysates when compared to KO. Significant reduction in frequency (f), expiratory time (Te), and inspiratory time (Ti) in KO animals was observed at 5mo of age upon hypercapnic challenge; however, KO+AAV animals maintained these parameters similar to WT. Conclusion. Neonatal intravenous administration of AAV-GAA improves respiratory capacity and ultimately lifespan in GAA knockout rats. Significant improvement in respiratory function and reduction of glycogen content are observed 5 months post-vector administration. The Pompe rat provides a novel model to examine pathologic disease mechanisms and evaluate the therapeutic potential of existing and next-generation therapies for Pompe disease.

357. Systemic Dose Escalation Study of Alpha-Sarcoglycan Provides Functional Improvement in *SGCA*$^{-/-}$ Mouse Model of LGMD2D

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Introduction: Mutations in the alpha-sarcoglycan (*SGCA*) gene cause a muscle disorder known as Limb Girdle Muscular Dystrophy Type 2D (LGMD2D). The loss or reduction of the alpha-sarcoglycan (αSG) protein leads to muscle weakness that affects proximal muscles, then spreads distally, resulting in impaired gait. Concomitantly muscle necrosis and elevated serum creatine kinase (CK) is characteristic. **Methods:** To correct the underlying histopathology and functionality of the muscle, we developed a self-complementary adeno associated virus (AAV) construct designed to carry the corrected *SGCA* gene to all muscle groups through a single systemic injection. A pre-clinical, three-dose escalation study (5×10^{13} vg/kg, 1×10^{14} vg/kg, 2×10^{14} vg/kg) was performed via tail vein delivery to *SGCA*$^{-/-}$ mice using scAAVrh74. SGCA. **Results:** All three doses exhibited significant improvement in diaphragm and tibialis anterior muscle strength and resistance to fatigue using ex-vivo physiology analysis. Also noted was an increase in mobility and activity in open-field analysis, and CK was reduced with all three doses. Histological exams of treated muscles showed a normalized and evenly distributed myofiber diameter with a reduction in both fibrotic collagen content and centralized nuclei. To confirm gene expression, immunofluorescence stains and western blots were performed; both assays showed restoration of gene expression comparable to wild-type levels at all three doses. **Conclusions:** Clinical trials and non-human primate studies using this scAAV. SGCA construct via intramuscular and isolated limb perfusion (ILP) delivery methods have demonstrated safety and efficacy. This dose escalation systemic delivery study in the *SGCA*$^{-/-}$ mouse model restored SGCA gene expression and improved motor function, providing proof of principle for one-time systemic delivery of AAV.SGCA in LGMD2D patients.

Gene Editing, Gene Delivery and Vector Design

358. Probing Capsid Mosaics Formation in Adeno-Associated Virus Library Preparations

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Barcoded adeno-associated virus (AAV) libraries are a powerful tool to investigate the complex relationship between the structure and biological functions of the AAV capsid. Virus libraries are produced by transfection of HEK293 cells with pools of barcoded, ITR-containing Rep/Cap plasmid variants. Their phenotypic diversity can further be explored through NGS analysis of the distribution of barcoded AAV transgene variants in different experimental setups. However, this approach is valid only assuming low levels of cross-packaging and capsid mosaicism. To date, the study of mosaic AAV formation has been carried out using indirect characterization methods, such as binding or infectivity assays. We and others have recently showed that capsid thermal stability measured by Differential Scanning Fluorimetry (DSF) was a reliable tool to probe the VP3 composition of AAVs in their virion conformation. In the present work, we used this biophysical approach to assess the impact of the production conditions on the structural homogeneity of AAV libraries. HEK293 cells were co-transfected with decreasing amounts of Rep/Cap plasmids encoding the VPs of both AAV8 and Anc82, two serotypes with very different melting temperatures (AAV8: 71°C and Anc82: 89°C) despite a relatively high VP3 sequence homology (93.83%). Viruses were purified by affinity chromatography, using the POROS CaptureSelect AAVX affinity resin, thoroughly characterized (qPCR, SDS-PAGE, Dynamic Light Scattering and TEM), and subjected to DSF. The resulting AAV libraries were pure, homogeneous in size, and followed the same VP1/2/3 1:1:10 stoichiometry. Yet, DSF profiles were closely dependent on the transfection conditions. The fluorescence signal of the library produced in saturating conditions (13 ug pRep/Cap per 15-cm dish) exhibited four distinct fluorescence transitions, indicative of AAV8 and Anc82 VP oligomerization (i.e. capsid mosaicism). Upon dilution of the plasmid library, fluorescence fingerprints progressively converged towards a reference signal, obtained by mixing homogeneous populations of AAV8 and Anc82, suggesting a gradual decrease in mosaic formation. Our approach emerges as a useful tool to study the process of VP oligomerization in the context of capsid assembly, and can be used to optimize the production conditions of AAV libraries.

359. Heterogeneic Genome Encapsidation of rAAV-CRISPR/Cas9 Vectors Underscores Potential Limitations for Promising *In Vivo* Gene-Editing Platforms

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The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system for gene editing has quickly become a realistic therapeutic platform for the permanent correction of genes associated with monogenic diseases. Many of these strategies now employ rAAVs as the vehicle of choice for delivering CRISPR/Cas9 components *in vivo*. With rAAVs on their own gaining momentum in clinical trials and having been granted FDA approval as the first human *in vivo* gene therapy drug, exploration of rAAV-mediated gene editing to treat disease has become more attractive than ever. These gains have necessitated the broadening of vector design approaches, preclinical investigation focused on therapeutic safety and efficacy of Cas9, and advancements to vector production pipelines to consider Cas9-related transgene components. Using AAV-Genome Population sequencing (AAV-GPseq), we recently demonstrated that rAAV vectors designed to deliver siRNA transgenes produced a high proportion of inert, truncated, and chimeric genomes. We surmised that the DNA structures of the siRNA cassettes, which exhibit high thermostabilities, promoted polymerase stalling and template-switching events during replication and genome packaging. In addition, the packaging of foreign DNAs tends to be centered at promoter regions of the host-packaging cell genome. Interestingly, these sequences do not share any clear distinguishing sequence motifs. Instead, hairpin-like structural features may promote the encapsidation of undesirable DNAs. Likewise, the development of quality control pipelines to query single-stranded rAAV-Cas9/sgRNA vectors is imperative. Using AAV-GPseq, we have now found that the single-guide (sg) RNA scaffold, which also harbors a degree of secondary structure, can also cause template-switching events. While we demonstrate that truncations specifically due to single unit sgRNA cassettes occur with much less frequency than with siRNA cassettes, the design of multiple guides in tandem worsens vector integrity. This preliminary work brings to light the importance of selecting and designing guide sequences when targeting gene editing events via rAAV vectors. Knowledge gained from these ongoing investigations will aid in the successful translation of rAAV-CRISPR/Cas9 and related platforms into the clinic.

360. Deep Search: Next-Gen Strategies for Accelerating AAV Capsid Engineering

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The complexity of AAV biology and the difficulty of predicting the functional effects of mutations have hindered efforts to engineer improved capsids for therapeutic applications. Most protein engineering methods to-date utilize some form of randomly generated diversity. These libraries are typically "shallow", containing few mutations per variant, since as more mutations are added the expected viability rapidly decreases. Here, we explore the potential of "deep" libraries containing up to 42 substitutions or insertions within a 28 amino acid region. We first tested the effects of >1000 single amino acid changes near the AAV2 heparin binding region on capsid assembly and biodistribution in mice, then systematically combined the resulting neutral and beneficial mutations to synthesize a deep library of 80,000 mutants. We found that randomly generated sequences quickly lost the ability to produce a viable capsid as they mutated further from the wild-type. Beyond four random mutations, more than 98% of randomly generated variants were deficient for capsid assembly. In contrast, deep libraries contained much higher levels of diversity (viable mutants with up to 10 mutations) while maintaining the ability to package and infect target tissues *in vivo*. Importantly, the library contained many capsids with greater delivery efficiency and tissue specificity, which can lead to safer and more effective therapies. This work highlights the benefits of using next-gen DNA synthesis and DNA sequencing for building and screening AAV libraries while demonstrating the potential of machine learning and data-driven protein engineering. Leveraging these emerging technologies will enable the field to design better AAV capsids that are safe, have low prevalence of pre-existing immunity, are efficiently produced, and are highly efficient and specific for target tissues

361. Compositional Control of *p*DNA/*l*PEI Nanoparticles Using Flash Nanocomplexation to Improve *In Vivo* Transfection Efficiency and Biocompatibility

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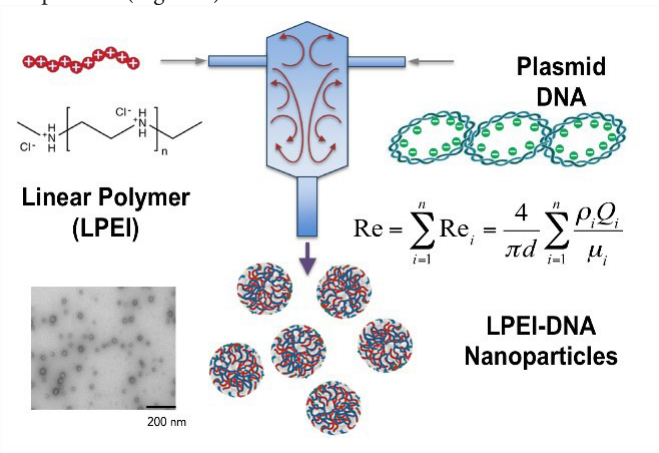
Linear PEI (*l*PEI) has proven track record as an effective carrier for the delivery of therapeutic plasmid DNA (*p*DNA) for disease treatment. However, the lack of a reproducible method to synthesize *p*DNA/*l*PEI nanoparticles with controlled composition and physicochemical characteristics has long hindered the development of these nanoparticles as a pharmaceutical product. We have employed turbulent mixing at high Reynolds numbers using the flash nanocomplexation (FNC) technique to achieve efficient and homogenous complexation of *p*DNA and *l*PEI, thus generating uniform nanoparticles with a well-defined composition (Figure 1).



We have shown that the operating parameters, including flow rate, pH, concentrations of all components, could be optimized to yield control in nanoparticle size, shape and surface property [1]. Here we investigated the feasibility of using the FNC technique to control the composition of *p*DNA/*in vivo*-jetPEI® nanoparticles by adjusting the feeding composition of *p*DNA and *in vivo*-jetPEI® in the reaction mixture, i.e. different N/P ratios. Since complexation under FNC conditions is kinetically controlled instead of equilibrium driven, the composition of the nanoparticles and the fraction of non-complexed *in vivo*-jetPEI® in the final suspension were well controlled. FNC-generated nanoparticles showed >40-fold increase in transgene expression in the lung of Balb/c mice when the N/P ratio was increased from 3 to 4. The expression of a transgenic luciferase reporter following transfection by these N/P4 FNC nanoparticles was comparable to that from nanoparticles generated by bulk mixing method prepared at N/P6, the formulation recommended by the *in vivo*jetPEI® manufacturer (Polyplus). Importantly, using the lower N/P ratio with the FNC method resulted in a significantly lower free *l*PEI in the final formulation and correlated with a significant reduction of toxicity in a BALB/c mouse model, in comparison with N/P6 bulk-mixed nanoparticles. These results demonstrate that the FNC technique, by optimizing the composition of the *p*DNA/*l*PEI nanoparticles, holds great potential to improve both *in vivo* transfection efficiency and biocompatibility, thus enabling the clinical translation of these nanoparticles as a delivery method for gene therapies. **Reference:** [1] J. L. Santos *et al.*, "*Continuous production of discrete plasmid DNA-polycation nanoparticles using flash nanocomplexation*," **Small**, 12 (45): 6214-6222 (2016). **Acknowledgements:** This study is partially supported by a research contract from Cancer Targeting Systems, Inc. (CTS) and an NIH grant R01EB018358. The authors thank Chris Ullman and Christine Carrington of CTS for helpful discussions.

362. CRISPR/Cas9-Based Whole Genome Screenings of Packaging Cells to Identify Cellular Factors for Enhanced rAAV Production

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Due to numerous advantages over other delivery options, recombinant AAV (rAAV) has emerged as a preferred vector for use in many human gene therapy strategies. However, the extreme cost associated with rAAV production greatly limits the clinical translation and commercialization of this promising viral gene therapy. We hypothesized that identification and modulation of cellular factors crucial for vector genome replication and/or packaging may enhance quality and yield of rAAV production. Thus, we have designed a CRISPR/Cas9-based whole-genome screening strategy for the identification of such cellular host factors. Gene- or promoter-targeting guide RNA (sgRNA) libraries, each consisting of >120,000 unique sequences, were constructed within rAAV vector genome plasmids enabling their delivery to packaging cell lines for rAAV production by different methods. Through initial screenings and high-throughput sequencing of rAAV-gRNA libraries produced by HEK-293 triple transfection, we demonstrate full plasmid library transfer and amplification to viral vectors. Sequencing packaged rAAV genomes at depth provided good library coverage with >300 reads per sgRNA, thus linking plasmid-based delivery of sgRNA to efficient viral vector production while maintaining library diversity. Furthermore, by packaging these rAAV sgRNA libraries within 293 cells engineered to express CRISPR/Cas9 activation or inactivation systems, strong statistical significance (p-values < 0.001, FDR < 0.25) is observed for both activation and inactivation of certain cellular genes. As library construction is robust and cost-effective, iterative rounds of screening and enrichment enable us to further enhance confidence in genes selected for downstream validation with experiments currently underway. Thus, by targeted screening of packaging cells during rAAV production, we present a novel and potentially versatile strategy to identify candidate cellular genes which may facilitate producer cell engineering efforts for increased packaging efficiency of rAAV production, possibly from a variety of vector production platforms.

363. A High-Throughput Method of Constructing and Screening Short Synthetic Gene Regulatory Elements

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Through advances in capsid design, adeno-associated virus (AAV) gene therapy is rapidly being optimized for enhanced delivery and greater specificity in tissue targeting. Although these advances reinforce the appeal for using AAV for targeted gene delivery, the small genome delivery size limits the capabilities of AAV as a comprehensive delivery vector. One way to effectively increase the AAV gene delivery capacity is to develop shorter enhancers that maintain the high transcriptional strength of conventional enhancers, thus leaving more room for the delivered gene of interest. We have built on a previous study's success, which synthetically optimized enhancers in the Chinese hamster ovary (CHO) cell line expression system, and have further increased the through-put and will screen our synthetic enhancers in an additional cell line and *in vivo* in mice. To create diverse and short enhancers, we generated a library of enhancers consisting of shuffled transcription factor binding sites (TFBS) constructed with a modular, randomized design. Oligonucleotides containing prominent TFBS elements (CCAAT-enhancer binding protein alpha (C/EBPa), Enhancer box (E-box), GC-box, Nuclear factor kappa B (NFκB)) were randomly assembled at a previously optimized ratio (C/EBPa: 10%, E-box: 30%, GC-box: 10%, NFκB: 50%) and ligated in the presence of oligonucleotides containing PstI restriction sites, to allow for control of enhancer length. These enhancers were cloned upstream of the minimal human cytomegalovirus (CMV) immediate early 1 core promoter into our barcoded plasmid library. This barcoded library approach will allow for specific detection of delivered genes, as well as their resulting transcripts, by Illumina deep sequencing to detect the associated barcodes amplified following DNA and RNA isolation. Single molecule real time (SMRT) sequencing was performed in order to categorize each randomized TFBS configuration with its associated barcode. In addition, barcoded plasmid controls were developed, either containing the entire CMV enhancer or just the CMV core promoter, for comparing the effectiveness of our synthetic enhancer library. Two cell lines, CHO-K1 and human embryonic kidney (HEK) 293 cells, were chosen for studying the enhancer impact. The randomized enhancer library was transfected into these cell lines for characterization of delivery and expression. In addition, the expression of the plasmid library was examined *in vivo* by hydrodynamic tail vein injection in mice, a technique that allows for efficient delivery of DNA to the liver. Target gene delivery and expression will be assessed in DNA and RNA isolated from liver samples obtained from mice after 4 days, 2 weeks, and 6 weeks post-injection of the shuffled enhancer plasmid library. In these cells and *in vivo* systems, gene delivery will be monitored by polymerase chain reaction (PCR) of isolated DNA and steady state transcription monitored by reverse transcription-PCR of isolated RNA. Our studies serve as a proof-of-principle for the rational design of new, shorter enhancers to aid in increasing the delivery gene capacity of AAV. We have begun by optimizing enhancers paired with the minimal CMV core promoter, but we anticipate that this technique can be further applied to cell-type specific enhancers. When applied to reducing the length of cell-type specific enhancers, this technique will not only select for high transcriptional activity from shorter enhancers, but also allow for an additional level of transcriptional specificity.

Preclinical Approaches in Gene Therapy for Neurosensory Disorders

364. Allele-Specific Deafness Gene Disruption through Discrimination of a Single Base Change by *S. aureus* Cas9^{KKH} Prevents Progressive Hearing Loss after AAV Mediated Gene Delivery

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Introduction: Single nucleotide changes in over 30 genes can cause dominant forms of hereditary hearing loss. Several strategies can be used to inactivate the disease-causing allele, such as siRNA, miRNA or genome editing. However, distinguishing mutant from wild-type alleles in the case of single nucleotide changes remains challenging. Here we explored several strategies to overcome the effects of a dominant M412K mutation in *Tmc1* using the Beethoven (*Bth*) mouse model. *Bth* animals are characterized by progressive hair cell degeneration and subsequent loss of hearing. ***Methods***: First, we established a fibroblast line from neonatal *Tmc1*$^{Bth/+}$ and *Tmc1*$^{+/+}$ animals. Fibroblasts were transfected with plasmids using electroporation. We designed different *S. pyogenes* and *S. aureus* CRISPR/Cas9 systems to target the mutation using four different strategies (n=14 variations): (1) changing the protospacer-adjacent motif (PAM) position in relation to the mutation site, (2) truncating gRNAs to allow more specific binding, (3) using a variant Cas9, derived from *S. aureus* (Cas9^{KKH}), (4) truncating gRNAs and using the *S. auerus* Cas9^{KKH}. We selected a Cas9^{KKH} variant, that has a PAM site specific for the *Bth* mutation, and is not present in the wild-type sequence. Allele-specific cutting was quantified by deep sequencing. Next, we injected AAV^{Anc80}-CMV-Cas9^{KKH}-U6-gRNA^{Bth} into the inner ears of heterozygous neonatal *Bth* mice. To test hearing after injection, we performed auditory brainstem recordings (ABR) at 4, 8 and 12 weeks post injection. We quantified the percentage of surviving hair cells at 12 weeks after injection using hair-cell-specific myosin-VIIa staining. With targeted deep sequencing, we analyzed allele-specific genome editing 7 days after injection. ***Results***: In fibroblasts, deep sequencing of the *Tmc1* gene revealed that none of the *S. pyogenes* Cas9-gRNA designs targeted the *Bth* mutation with high specificity, as we observed genome editing on both the mutant and the wild-type *Tmc1* alleles. With *S. pyogenes* Cas9, a truncated gRNA produced the highest *Bth*/WT relative specificity ratio (81.9%). However, the *S. aureus* Cas9^{KKH} with a truncated gRNA yielded essentially no WT allele cutting, but led to insertion and deletion (indel) formation on the *Tmc1*^{Bth} allele (relative specificity ratio of 99.8%). In heterozygous *Bth* mice, injection of AAV^{Anc80}-CMV-Cas9^{KKH}-U6-gRNA^{Bth} led to a robust improvement of hearing thresholds, with some animals exhibiting near normal hearing sensitivity (in contrast to uninjected animals, which have severe-to-profound hearing loss), and hearing sensitivity was stable over time out to 12 weeks, the latest time point tested. Sequencing from *in vivo* injected samples revealed allele-specific indel formation in the *Tmc1* gene. The most common genetic changes were deletions and integration of the AAV cassette itself into the *Tmc1*^{Bth} gene, both disrupting the reading frame. ***Conclusion***: AAV delivery of the mutant *S. aureus* Cas9^{KKH} can disrupt an allele bearing a point mutation, with high specificity based on PAM site recognition, leading to efficient prevention of hair-cell degeneration and arrest of progressive hearing loss. Optimal CRISPR design is important in targeting single-nucleotide changes. AAV-mediated *in vivo* genome editing holds promise for *Tmc1* and other forms of dominant progressive hearing loss. ***DPC and JRH contributed equally.***

365. A Single Neonatal Injection of PHP.B-AAV9-*Clrn1* Rescues Hearing in a Model of Usher Syndrome Type IIIa

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Introduction: Usher syndrome type IIIa is characterized by progressive hearing loss and retinal degeneration and affects at least 1 in 800,000 people. It is caused by a loss-of-function mutation in the clarin-1 (*CLRN1*) gene resulting in autosomal recessive inheritance. Here we characterized the phenotype of *Clrn1* KO mice and tested whether a neonatal injection of an AAV vector encoding clarin-1 improves hearing. ***Methods***: We analyzed the expression of mouse *Clrn1* isoforms in the cochlea at different stages (embryonic day 18 to postnatal day 7 (E18 to P7)) by RT-PCR. In *Clrn1* knock-out and heterozygous animals (gift from Dr. Kumar Alagramam), we next evaluated stereocilia morphology by microscopy, hair cell function by FM1-43 uptake and hearing function by auditory brainstem recordings (ABR). For gene addition, we packaged isoform 2 of *Clrn1* in AAV9-PHP.B vector, a previously described capsid for CNS transduction that we found to efficiently transduce inner and outer hair cells after round window membrane injection of neonatal pups. We also created N-terminal and C-terminal hemagglutinin (HA) tagged *Clrn1* constructs to allow immunostaining to localize the clarin-1 after viral gene delivery. Finally, we used ABR to test hearing at 4 and 9 weeks after injection of the vector to assess efficiency of gene addition. ***Results***: Based on RT-PCR expression we found that isoform 2 (lacking exon 2) is the predominant isoform of *Clrn1* in both the organ of Corti and the spiral ganglion region from E18 to P7. Isoform 3 (lacking exons 2 and 3) was also expressed, albeit at lower levels. We found that bundle orientation and morphology was disturbed in KO animals compared to heterozygous littermates as early as postnatal day 0, similarly to previous reports (Geng R. et al. 2012). FM1-43 uptake was present, but significantly decreased, in inner and outer hair cells of KO animals compared to heterozygous animals at P5. ABR measurements revealed highly elevated thresholds in the KO animals at P25, with minimal residual hearing in 2/8 ears (8 to 22.6 kHz, lowest threshold of 80 dB, undetectable in other animals). Injection of AAV encoding N-HA-*Clrn1* at P1 resulted in expression of the protein in the stereocilia and cuticular plate of both inner and outer hair cells (~90% of IHC and ~40% of OHC). Four weeks after injection, ABR reported detectable

hearing thresholds in 10/17 animals with the lowest thresholds of 50 dB at 4 and 8 kHz. ABR thresholds were significantly improved in the lower frequencies in injected KO vs. non-injected KO animals ($p<0.05$ at 4, 5.6, 8, 11 kHz); while no statistically significant hearing improvement was seen at higher frequencies (16, 22, 32 and 45 kHz). Hearing thresholds were also significantly improved on the injected (left) side vs. the non-injected ear (right) of in the same animal at lower frequencies ($p<0.05$ at 4, 5.6 and 8 kHz, not significant for higher frequencies). ABR in injected animals was still detectable at 9 weeks post-injection. ***Conclusion***: Mice lacking *Clrn1* are characterized by disorganized hair cell bundles and ultimately profound hearing loss. A single neonatal injection of AAV9-*Clrn1* improves hearing at lower frequencies in *Clrn1* KO animals. Further studies are necessary to evaluate gene therapy efficiency in adult animals using better models. Our results suggest gene addition holds promise as a therapy for Usher syndrome type IIIa.

366. Peripheral Gene Therapeutic Rescue of an Olfactory Ciliopathy Induces Central Neural Plasticity and Restores Odor Guided Behavior

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Ciliopathies are a class of inherited pleiotropic genetic disorders that manifests from the disruption of cilia assembly, maintenance, and/or function. Due to the ubiquitous role of cilia in cellular signaling and homeostasis, ciliopathies often affect multiple tissues and organ systems, including the olfactory system. Odor detection initiates within cilia of olfactory sensory neurons (OSNs) where loss of ciliation result in olfactory dysfunction or anosmia. Although therapeutic strategies are limited, vector-mediated expression of wildtype genes had demonstrated the ability to restore ciliation in ciliopathy models. However, it remains unclear whether the restorative capacity of vector-mediated expression could extend to neuronal processing and central olfactory system. In order to address this, we developed an OSN specific IFT88 knockout mice (*Ift88osnKO*) to examine the mechanisms of ciliopathy-induced olfactory dysfunction and the potential for gene replacement to induce plasticity, repair olfactory circuitry, and restore odor-guided behaviors. Loss of OSN cilia resulted in substantially reduced odor detection and odor-driven synaptic activity at the level of the olfactory bulb (OB). Defects in OSN axon targeting to the OB were also observed in parallel with aberrant odor-guided behavior. Intranasal adenoviral gene delivery of wild-type IFT88 to *Ift88osnKO* mice rescued OSN ciliation and peripheral olfactory function. Importantly, this recovery of sensory input in a stochastic subset of terminally differentiated OSNs was sufficient to induce plasticity and restore axonal targeting in the OB of juvenile mice. In addition, restoration was recapitulated in adult mice, albeit demonstrating delayed onset of rescue. Furthermore, restoration of sensory input re-established appetitive and aversive odor-guided behaviors. Overall, these findings highlight the spare capacity of the olfactory epithelium and the plasticity of primary synaptic input into the central olfactory system. The demonstrated restoration of peripheral and central neuronal function suggests the therapeutic potential of ciliopathy-related and other gene-associated anosmia using gene therapy. This work was supported by the National Institutes of Health R01DC009606 to J.R.M.

367. Gene Augmentation Therapy in a Large Animal Model of *PDE6A*-Retinitis Pigmentosa Rescues Rod Function and Promotes Cone Survival Long-Term

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Purpose: Assessment of long-term functional rescue and structural preservation of translatable AAV-mediated retinal gene augmentation in a large animal dog model of *PDE6A* retinitis pigmentosa. PDE6A is expressed in rod photoreceptors and mutations cause a lack of rod phototransduction, a rapid loss of rods followed by a slower secondary loss of cones. Having previously shown restoration of rod function in short-term studies, we wanted to investigate rod as well as cone rescue and structural preservation of both photoreceptors in the longer term. **Methods:** An adeno-associated viral vector serotype 8 delivering human *PDE6A* cDNA under control of a short rhodopsin promoter (AAV8-h*PDE6A*) was injected subretinally in 4 *Pde6a*-/- puppies at a dose of 5.10^11 vector genomes (4 eyes had successful subretinal bleb, 2 eyes were uninjected and used as controls, and 2 eyes were excluded as injections were suboptimal with small and rapid bleb flattening). *In vivo* outcome measures included vision testing (VT) using a four-choice vision testing device at light levels covering scotopic, mesopic and photopic vision, electroretinography (ERG) and spectral domain-optical coherence tomography (SD-OCT) up to 8 months post injection. **Results:** Therapy restored rod function to treated eyes as assessed by both ERG and VT. When using treated eyes dogs could accurately choose the correct exit tunnel and rapidly exit the device at the lowest light levels which assess rod only function. Dark-adapted ERGs showed a dramatic improvement of response threshold and wave form amplitudes. Functional rescue was maintained for the duration of the study. As the dogs got older a difference in cone-mediated ERG amplitudes became apparent between the treated eyes and control untreated eyes. SD-OCT showed normal appearing zones (discrete external limiting membrane, ellipsoid and interdigitation zones recognizable) representing the photoreceptor inner and outer segments in the treated retinal region. It also showed halting of the thinning of outer retinal layers with preservation of the Receptor Plus (REC+), Outer nuclear layer (ONL) and total retina (TR) thicknesses in the treated region in all puppies up to 8 months PI ($P<0.01$). No significant changes in the inner retina (IR) thickness were noted. **Conclusions:** Follow-up of outcome measurements *in vivo* up to 8 months PI showed maintained rod photoreceptor functional rescue and preservation of retinal structure. Cone photoreceptor function as assessed by ERG was maintained in the treated eyes whereas it underwent progressive loss in the untreated eyes. Use of the translatable AAV8-h*PDE6A* vector to achieve long-term rescue of function and preservation of structure in this clinically relevant large-animal model is an important step towards

human clinical trials to treat *PDE6A*-RP. Acknowledgments/Funding: Myers-Dunlap Endowment for Canine Health, RD-Cure consortium and Tistou & Charlotte Kerstsan Stiflung

368. Effect of Inducible VEGF-Trap Expression on CNV Formation in a Murine Model of Wet AMD Following Intravitreal Administration of a Capsid Mutant rAAV-Riboswitch Vector

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Purpose: Age-related macular degeneration (AMD) is one of the leading causes of vision loss in the developed world. In 15% of patients, AMD progresses to an exudative stage characterized by chorodial neovascularization (CNV) and loss of central vision. Development of CNV is triggered largely through extracellular vascular endothelial growth factor A (VEGFA) signaling. consequently, current treatments for wet AMD are based around repetitive administration of VEGF traps (e.g. Eylea). Although anti-VEGF treatments have proven to be extremely effective at preventing CNV**,** continuous anti-VEGF exposure over a period of years has been shown to cause increased retinal pigment epithelium (RPE) and photoreceptor atrophy, leading to a decrease in visual acuity. Here, we evaluate the efficacy of a tetracycline responsive 'OFF-type' riboswitch (TC45) in regulating Eylea expression in the mouse retina following recombinant adeno-associated viral (rAAV) delivery. **Methods**: Six-week-old C57BL/6J mice were intravitreally injected with PBS or $5.0x10^{10}$vg of rAAV encapsulating either a constitutively active (smCBA-Eylea) or a tetracycline-inducible (smCBA-Eylea-1x-TC45) Eylea construct. smCBA-Eylea-1x-TC45 injected mice were placed in two groups with mice receiving standard diet (ON-state; high levels of Eylea expression) or tetracycline diet (OFF-state; reduced Eylea expression). CNV was induced using an 810nm diode laser six weeks following injection. Seven days following laser injury, CNV formation was evaluated using fluorescein angiography with leakage independently graded by three blinded individuals. Finally, eyes were harvested and the concentration of Eylea was determined by ELISA. **Results:** Constitutive expression of Eylea (smCBA-Eylea) significantly reduced the severity of CNV formation and leakage compared to PBS sham injected eyes ($p<0.0001$). Moreover, smCBA-Eylea-1x-TC45 injected mice receiving standard diet (Eylea expression 'ON') had a significantly reduced number of clinically significant lesions compared to smCBA-Eylea-1x-TC45 injected mice receiving tetracycline diet (Eylea expression 'OFF') ($p=0.0008$). Tetracycline mediated activation of the TC45 riboswitch resulted in a significant decrease in Eylea concentration in smCBA-Eylea-1x-TC45 injected mice ($p<0.05$). **Conclusions:** Over-expression of Eylea in the mouse retina following rAAV delivery significantly reduced the severity of CNV formation. Furthermore, tetracycline mediated TC45-activation significantly reduced the expression of Eylea, resulting in an increase of clinically significant lesions. In summary, riboswitch mediated regulation of Eylea opens the door for the development of a personalized gene therapy strategy for the treatment of wet AMD. Ultimately, we propose that neovascularization in wet AMD can be controlled following a single administration of an rAAV.riboswitch.anti-VEGF vector that allows for periodic over-expression of a VEGF trap simply through dosing of the activating ligand (e.g. an oral drug). This strategy would effectively eliminate the need for invasive, repetitive (i.e. monthly) intra-ocular injections, greatly improving treatment safety and patient quality of life.

369. Hypoxia-Regulated, Cell-Specific Angiostatic Gene Therapy for Choroidal Neovascularization (CNV)

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Choroidal neovascularization (CNV) is one of the characteristic features of age-related macular degeneration (AMD) that causes severe visual loss among the elderly. Pro-angiogenic factors like vascular endothelial growth factor (VEGF) play an important role in the onset and progression of CNV, defined as growth of new vessels from choroidal capillaries into the sub-retinal pigment epithelial (RPE) space or retinal layers. Ocular gene therapy delivering angiostatic agents could avoid repeated injections but long-term suppression of VEGF in the retina may remain problematic. Regulation of the transgene by the physiological condition avoids these potential problems, and offers rapid targeting of recurrence of CNV. With that goal in mind, we developed a hypoxia-regulated gene therapy to drive expression of a therapeutic gene product in the same hypoxic/inflammatory conditions that drive VEGF expression and growth of CNV. Our new AAV2 vector (AAV2-HRSE-6XHRE-RPE65-Endo) incorporated the RPE-specific promoter and HIF-1 response elements (HRE) to deliver human endostatin (an angiostatic protein) to only those RPE cells in hypoxic foci. We used the mouse model of laser-induced choroidal neovascularization (CNV) to evaluate the prophylactic effectiveness of this vector. Control (AAV2-CMV-GFP) and experimental (AAV2-HRSE-6XHRE-RPE65-Endo) vectors were injected subretinally into C57BL/6J mice eye, and laser photocoagulation was used to induce experimental choroidal neovascularization 21 days or 120 days later. Choroidal neovascular area in vector treated and vehicle-control eyes was analyzed 14 days after laser treatment by spectral domain optical coherence tomography (SD-OCT) and RPE flat mounts Quantitative RT PCR was used to measure exogenous endostatin expression in RPE/Choroid tissues at 3, 7, 14, and 45 days following laser photocoagulation and compared with the unlasered group. Exogenous endostatin expression by the hypoxia-regulated vector was significantly elevated 3, 7 and 14 days following laser burn and its expression was shut down by 45 days. Spectral domain optical coherence tomography (SD-OCT) images from live mice and confocal images from lectin stained RPE flat mounts demonstrated that CNV area was reduced by 80% compared to untreated eyes ($P<0.001$). The development of RPE-specific, hypoxia-regulated gene therapy delivering angiostatic agents could be a valuable therapeutic approach to reduce neovascular AMD at the time and in the space where it arises.

Immune Cell Therapies

370. Restraining Macrophages Alleviates CAR T Cell-Induced Cytokine Release Syndrome and Informs Novel Therapeutic Interventions

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CD19 chimeric antigen receptor (CAR) therapy is highly effective for some refractory B cell malignancies. Its broad implementation in non-specialized healthcare facilities and potential to be administered as first-line therapy is limited in part by the occurrence of severe cytokine release syndrome (CRS). CRS presents with fever, hypotension and respiratory insufficiency, which can result in multi-organ failure. This pathology is accompanied by elevated serum cytokines including interleukin-6 (IL-6). Blockade of IL-6 can abate CRS in some but not all patients, for which lymphotoxic doses of corticosteroids are needed. The physiopathological mechanisms of CRS are currently unknown and thus there is an imperative need for animal models to delineate the identity and specific roles of the cell populations involved. Ultimately, animal models will be indispensable as a guide towards the rational design of effective treatment approaches that do not compromise the therapeutic efficacy of CAR T cells. We have established a novel mouse model in which CD19 CAR T cell-induced CRS can be reliably elicited, paralleling the rapid onset and acuteness observed in clinical trials. Symptoms present within 24 hours after CAR T cell administration, followed by rapid weight loss and eventual mortality. The cytokine profile of this mouse model remarkably mirrors that of clinical CRS (18/19 cytokines). Importantly, IL-6 is of host origin and not CAR T cell derived. Lastly, treatment with IL-6R blockade prevents CRS-associated mortality, in accordance with the demonstrated clinical benefit of tocilizumab. We further show that CRS is a tripartite pathology elicited by the interaction of tumor cells, CAR T cells and myeloid cells. CAR T cell-tumor interaction leads to a robust recruitment of myeloid cells to the tumor site, including neutrophils, monocytes and macrophages. Transcriptome analyses reveal that myeloid populations proximal to tumor and CAR T cells, but not in distal sites such as the spleen, are activated and responsible for IL-6 production. Furthermore, by selectively activating macrophages through the CD40L pathway, we show a significant increase in cytokine production and CRS severity. Conversely, the inhibition of core macrophage functions prevents mortality and mice recover faster from CRS toxicity. Overall, our results suggest an indispensable role for the myeloid system in severe CRS, especially macrophages, representing a cellular compartment that has been largely overlooked in pre-clinical models to date. Our findings on macrophage engagement in CRS identify multiple therapeutic interventions that can ameliorate the toxicities of CRS, in part, by targeting macrophage functions. Our model should be useful to guide the rational design of CAR T cells that can autonomously ameliorate or prevent CRS, eventually obviating the need for external pharmacological intervention.

371. Development of an Allogeneic Nkt Cell Platform for Off-The-Shelf Cancer Immunotherapy

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Current cancer immunotherapy clinical protocols involving autologous or HLA-matched allogeneic polyclonal T cells require patient-specific cell manufacturing processes that produce substantial variability and high costs of treatment. In contrast to HLA-restricted T cells, CD1d-restricted NKT cells (NKTs) are not alloreactive, and as a result could be used to treat multiple patients without the risk for graft-versus-host disease. Despite this advantage, HLA-mismatched NKTs would still be recognized as foreign and targeted for elimination by the host immune system. To overcome this challenge and prolong the persistence of therapeutic NKTs in an allogeneic host, we targeted both β2-microglobulin (B2M) and the MHC class II-associated invariant chain (Ii), and by extension expression of HLA class I and II in *ex vivo* expanded NKTs from healthy donors. We found that both complete elimination of B2M expression via CRISPR-mediated gene deletion and graded downregulation of B2M and/or Ii using shRNA-mediated RNA interference were effective in reducing NKT-induced stimulation of allogeneic CD8 T cells and CD4 T cells, respectively. Surprisingly, a majority of NKTs remained resistant to allo-NK cell cytotoxicity after B2M knockout or B2M and/or Ii knockdown. Finally, we engineered B2M- and Ii-specific shRNAs to be expressed together with cDNA for a tumor-specific chimeric antigen receptor in a single retroviral construct for one-hit generation of tumor-specific, universally tolerated NKTs for off-the-shelf cancer immunotherapy.

372. A Phase I Clinical Trial with *Ex Vivo* Expanded Recipient Regulatory T Cells in Living Donor Kidney Transplants

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There is considerable interest in therapeutic transfer of regulatory T cells (Tregs) for controlling aberrant immune responses. Initial clinical trials have shown the safety of Tregs in hematopoietic stem cell transplant recipients and subjects with juvenile diabetes. Our hypothesis is that infusion(s) of Tregs may induce transplant tolerance thus avoiding long-term use of toxic immunosuppressive agents that cause increased morbidity/mortality. Towards testing our hypothesis, we conducted a phase I dose escalation safety trial infusing billions of *ex vivo* expanded recipient polyclonal Tregs into living donor kidney transplant recipients. Despite variability in recipient's renal disease, our expansion protocol produced Tregs which met all release criteria, expressing >98% $CD4^+CD25^+$ with <1% $CD8^+$ and $CD19^+$ contamination. Our product displayed >80% FOXP3 expression with stable demethylation in the FOXP3 promoter. Functionally, expanded Tregs potently suppressed alloresponse and induced the generation of new Tregs in the recipient's allo-responders *in vitro*. Within recipients, expanded Tregs amplified circulating Treg levels in a sustained manner.

Clinically, all doses of Treg therapy tested were safe with no adverse infusion related side effects, infections or rejection events up to two years post-transplant. This study provides the necessary safety data to advance Treg cell therapy to phase II efficacy trials.

373. Control of Human T-Cell Expansion by Chemically-Induced Signal Complexes

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Adoptive T cell therapies have tremendous potential for the treatment of cancer and autoimmune diseases. The ability to control the proliferation of therapeutic cells after transplantation could enhance the efficacy of these therapies, including preventing patient toxicities and improving cell persistence. With the goal of engineering T cells that could be inducibly expanded *in vivo* by administration of a clinically-relevant drug, we designed a synthetic cell surface receptor to provide an IL-2 growth signal in the presence of Rapamycin, which we have termed a CISC (Chemically-Induced Signal Complex). The CISC requires the co-expression of two proteins: the rapamycin binding domains of FKBP50 or mTOR (termed the FRB domain) fused to the transmembrane and intracellular domains of IL2Rγ or IL2Rβ, respectively. We used protein structure modeling to design several candidate architectures to try to optimize the ability of Rapamycin to dimerize the FKBP and FRB domains, and induce the proximity of IL2Rβ and IL2Rγ intracellular domains (and thus mimic an IL-2 receptor signal). Using lentivirus to transduce primary human T cells, we identified an architecture that provided substantial expansion in cytokine-free culture media containing rapamycin or an alternative 'rapalog' compound. Flow cytometry analyses demonstrated the capacity of the drug-induced CISC signaling to selectively expand CISC-expressing cells within a mixed population. We next improved upon the expansion of CISC expressing cells in Rapamycin by co-expression of a third protein, an intracellular FRB domain, to act as a decoy for intracellular Rapamycin binding to mTor. While potential applications of CISCs are broad, in the current study, we focused our work on engineering Rapamycin-expandable human regulatory T cells (T_{reg}). Using HDR (homology directed repair)-based gene editing approaches previously developed in our laboratory, CRSPR/Cas9 and AAV6 donor templates targeted a donor cassette to the endogenous *FOXP3* locus to drive co-expression of CISC and FOXP3 in primary human T cells. As a proof-of-concept, this approach improved the enrichment and expansion of FOXP3 expressing gene edited (ed)T_{reg} cultured in Rapamycin, relative to nT_{reg} or edT_{reg} expressing FOXP3 alone. Further work will determine the immunosuppressive capacity of CISC expanded FOXP3$^+$ T cells *in vitro* and *in vivo*, and their persistence and proliferation in the presence of Rapamycin.

374. T Rapa Cells as Vehicles for Delivery of Therapeutic Cargo

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Hematopoietic stem cell (HSC)-directed gene therapy is being tested in amenable monogenic deficiencies. As an example, our lab is currently conducting a phase I clinical trial (NCT02800070) aimed at treating patients with Fabry disease (FD) by gene transfer. FD is an α-galactosidase A (α-gal A) deficiency in which globotriaosylceramide (Gb_3) and other metabolites accumulate. In our protocol, CD34$^+$ hematopoietic cells are transduced *ex vivo* with a recombinant lentivirus (LV) to engineer overexpression of α-gal A. These cells are then returned to the patient. Leukocytes derived from vector-transduced HSCs can secrete α-gal A and uncorrected cells can take it up, a process termed "cross-correction". For efficient engraftment of autologous cells, patients receive myeloid cell-depleting conditioning regimens that can cause significant morbidity. In addition, HSCs must be mobilized by various medication prior to apheresis or collected from BM aspirates. Alternative circulating cell populations that can be readily obtained without mobilization and can be engrafted with less toxic regimens may therefore be more desirable to use to deliver therapeutic cargo systemically. T cells are natural protein-secreting machines and are already employed in many clinical trials. Unlike HSCs, T cells can be obtained from peripheral blood (PB) without mobilization and can be expanded exponentially in culture. *Ex vivo* treatment with rapamycin elicits numerous changes in T cells that, in sum, endow them with an anti-apoptotic and pro-engraftment phenotype; as a result, such T Rapa cells can engraft with less host conditioning. Our labs have been investigating the potential for autologous CD4$^+$ T Rapa cells to function as "circulating micropharmacies" to deliver therapeutic transgene products systemically. We have shown that T Rapa cells can be manufactured from PB cells of affected FD patients and normal donors (ND) and can be productively transduced with the LV used in the Fabry clinical trial detailed above. After *in vitro* expansion for 2 weeks, transduced T Rapa cells continue to secrete α-gal A in the absence of stimulation. Transduced and control ND and FD T Rapa cells were then xenografted into NOD/SCID/Aga$^{-/-}$ mice (NSF). Higher plasma α-gal A activity was detected in mice given LV-modified cells. Vector copy number analyses suggest stable transduction. NSF mice receiving transduced cells also exhibited reduced plasma Gb_3 levels. We are determining enzyme activity and Gb_3 levels in other tissues of xenografted NSF mice to assess the level of "cross-correction" mediated by these delivery vehicles. We also plan to optimize rat-to-rat adoptive transfer of T Rapa cells to examine the feasibility/consequence of repeat administration and characterize the dynamics of transgene product

delivery. In parallel we are testing the ability of T Rapa cells to express and secrete proteins relevant in other monogenic deficiencies, such as Gaucher disease and Farber disease.

375. Scalable Generation of iPSC-Derived Macrophages Displaying *In Vivo* Plasticity

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Tissue resident macrophages (TRM) have recently been highlighted as key players in tissue homeostasis in various organs. They can be found as alveolar macrophages in the lung, microglia in the brain, Langerhans cells in the skin or Kupffer cells in the liver. Although most TRM populations possess stem cell like features and are able to maintain their population under homeostatic conditions, bone marrow-derived monocytes can replenish resident macrophage pools in case of organ damage or disease. This remarkable plasticity renders monocytes/macrophages an attractive population for diverse cell therapeutic approaches. Given the exceptional proliferation and differentiation capacity of induced pluripotent stem cells (iPSCs), we here explored the potential of human iPSCs as an (alternative) cell source for the generation of therapeutic macrophages in sufficient quantities to investigate novel cell therapies. In order to provide a scalable differentiation system, we first established a suspension-based hematopoietic differentiation protocol able to continuously generate human iPSC-derived macrophages (iPSC-Mac) from "myeloid cell forming complex (MCFC)" intermediates over several months. Upscaling of the differentiation process to an industry compatible, stirred tank bioreactor system resulted in the continuous generation of 20-30 million highly pure $CD45^+CD11b^+CD14^+CD163^+$ iPSC-Mac per week under defined conditions. Produced iPSC-Mac displayed a transcriptional profile similar to peripheral blood derived phagocytes, however, with a distinct signature towards primitive macrophages. Of note, iPSC-Mac demonstrated *in vitro* and *in vivo* plasticity and were able to respond to external/environmental stimuli. Thus, polarization of iPSC-Mac with M-CSF or GM-CSF resulted in the induction of a M2/M1-like phenotype, respectively. Moreover, after contact with *Pseudomonas aeruginosa*, iPSC-Mac remodeled their transcriptome towards the signature of activated macrophages and showed marked upregulation of cytokines (e.g. IL23A, TNFα, IL1A, IL6, IFNG1), chemokines (e.g. CCL5, CCL20, CCL4, CXCL3) and molecules involved in NFkB signaling. Even more importantly, iPSC-Mac adapted to the instructive tissue environment after intra-pulmonary transplantation into an immunodeficient mouse model as demonstrated by whole transcriptome analysis. In unbiased hierarchical cluster analysis as well as principal component analysis iPSC-Mac and BM derived macrophages re-isolated 2 month post transplantation clustered together with primary human alveolar macrophages and significantly differed from iPSC derived macrophages before transplantation. In summary, we demonstrate the scalable and continuous production of primitiv hiPSC-derived macrophages which are able to adapt and integrate to the local tissue environment and thus might offer an exciting cell source for novel macrophage-based therapies.

Therapeutic Intervention Using Tissue Directed Non-Viral Gene Transfer

376. Evaluating *In Vivo* Electroporation as a Method of Gene Transfer for Cardiac Gene Therapy

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Introduction: The atrial fibrillation (AF) disease state progresses via two distinct pathologies affecting the atrial myocardium: electrical remodeling and increasing fibrosis. Effective gene therapies against AF will target both of these pathologies, and as such necessitate the use of gene transfer strategies that can effectively deliver multiple transgenes to the atrial substrate. Electroporation is an inexpensive, non-immunogenic method of gene transfer that is capable of delivering naked DNA sequences in a mode independent of vector packaging size limitations. Recent research suggests that electroporation also enhances expression of viral vectors in the heart. These considerations therefore make electroporation an attractive gene transfer strategy for both non-viral and viral AF gene therapy. However, electroporation is difficult to perform *in vivo* in the intact atrium. Throughout a series of studies in a large animal dog model, we have demonstrated the feasibility of *in vivo* electroporation for sustainable gene transfer to the atrium in the beating heart. **Methods:** During thoracotomy, plasmids containing genes of interest (diluted to 2 mg/mL in sterile saline) were injected into the posterior left atrium (PLA) of dogs. 5-6 sub-epicardial 1 mL injections were made to ensure full PLA plasmid coverage. Immediately after each injection, 8 electrical pulses (200 V, 10 ms pulse length, 1s intervals) were delivered to the injection site via two gold-plated, needle style electrodes 1 cm in length, with a 1 cm inter-electrode distance. The chest was then closed and the dogs were allowed to recover. Three weeks after the initial procedure, dogs were sacrificed in a terminal study and tissue samples were obtained for transgene expression analysis. RT-qPCR, western blotting and immunohistochemistry were performed to elucidate the patterns of transgene expression. **Results:** Western blotting and immunohistochemistry revealed robust expression of transgene in tissues that had been targeted for electroporation. Importantly, similar levels of transgene expression were not noted in neighboring, un-injected regions of the atria, illustrating electroporation's ability to obtain highly localized gene transfer (figure 1). Furthermore, RT-qPCR analysis of heart tissue injected with varying concentrations of plasmid showed significant dosage dependence, with higher plasmid concentrations leading to

higher transgene uptake. Though most trials were terminated 3-4 weeks after gene injection, we have observed sustained expression of transgene up to eight months post injection. These results were not transgene specific; we saw similar levels of expression across a variety of different plasmids, even when co-injection of multiple plasmids was performed. **Conclusion:** Electroporation is a feasible and effective method of gene transfer in large animals. Despite this efficacy, the procedure itself remains technically difficult due to the need for sequential electroporation at multiple atrial sites. To combat this, we have been developing a, new, multi-electrode electroporation device that will allow us to perform simultaneous electroporation in multiple gene-injected sites over a large atrial surface area. This will help shorten and simplify the electroporation procedure, thereby making it more reproducible and user friendly for cardiac surgeons. Our data in conjunction with our plans for future device design make electroporation *in vivo* an attractive gene transfer method for gene therapy applications.

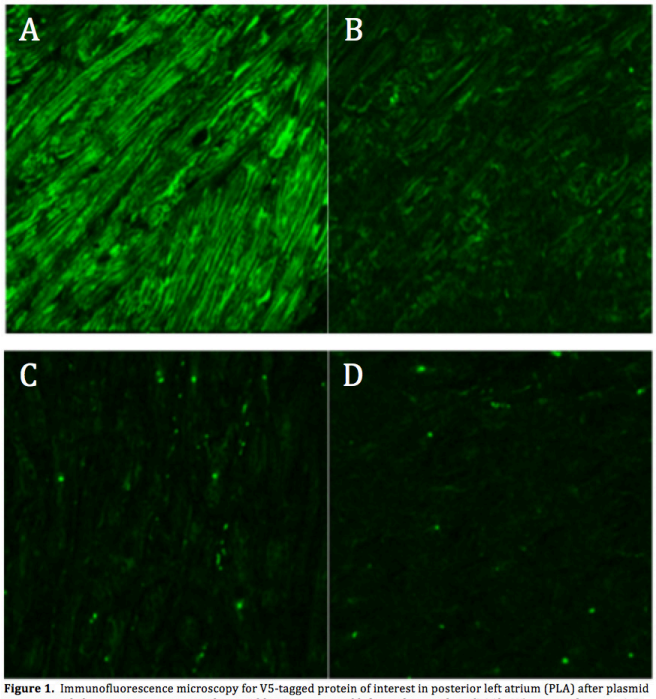

Figure 1. Immunofluorescence microscopy for V5-tagged protein of interest in posterior left atrium (PLA) after plasmid injection and electroporation compared to neighboring untreated left atrial appendage (LAA). V5 is stained green. A-B, Expression of V5-tagged protein in PLA after localized plasmid injection and electroporation. Protein expression is heterogeneous but robust. C-D, Expression of V5-tagged protein in neighboring LAA. Insignificant levels of protein expression are observed.

377. Targeted Homology Directed Repair in Blood Stem and Progenitor Cells with Highly Potent CRISPR Nanoformulations

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Retrovirus-mediated gene addition into blood stem and progenitor cells has demonstrated efficacy in inherited, malignant and infectious diseases. However, large-scale vector production and the inability to target the gene addition remain substantial limitations for retrovirus-based gene therapy. A non-viral, targeted gene delivery approach with the same transgene plug-and-play utility as retroviruses would be a major advance. Here, we have developed a highly potent, "fully-loaded" CRISPR nanoformulation capable of delivering both synthetic, non-chemically modified CRISPR Cpf1 or CRISPR Cas9 ribonucleoproteins along with a ssDNA donor template for insertion of new DNA, without the need for electroporation (Fig. 1A). Our multilayered nanoformulation had an average size of 30 nm and was highly monodisperse. Transmission electron microscope images (TEM) and localized surface plasmon resonance shifts (LSPR) of gold nanoparticles (AuNPs) showed a uniform surface coating without any aggregation (Fig. 1A, B). Given the synthetic nature of the entire delivery system, all components can be assembled in a few hours. Confocal microscopy demonstrated AuNP uptake into $CD34^+$ hematopoietic stem cells from healthy donors by simple AuNP addition to liquid cultures. We demonstrated knock-in frequencies of up to 10% at the *CCR5* locus using a *NotI* restriction enzyme template with homology arm lengths of ±40 nucleotides without cytotoxicity. Designing template to the non-target DNA strand yielded a higher homology directed repair (HDR) efficiency (Fig. 1C), evidenced by 447 bp and 316 bp cut bands following digestion with NotI or T7EI enzymes (Fig. 1D). Direct comparison of Cpf1 and Cas9 nuclease activity at the same CCR5 target site demonstrated a Cpf1 bias for increased HDR/NHEJ ratio as compared to the Cas9 nuclease. Xenotransplantation of CRISPR Cpf1 nanoformulation-treated human $CD34^+$ cells into sublethally irradiated immune deficient (NSG) mice demonstrated an early engraftment advantage at 4 and 6 weeks compared to non-treated cells, suggesting a possible engraftment advantage of nanoformulation-treated hematopoietic cells that requires further exploration. Studies to demonstrate the utility of this system for gene addition using larger templates are ongoing. This highly potent and safe "all-in-one" nanoformulation has tremendous potential in clinical applications of hematopoietic gene therapy and gene editing.

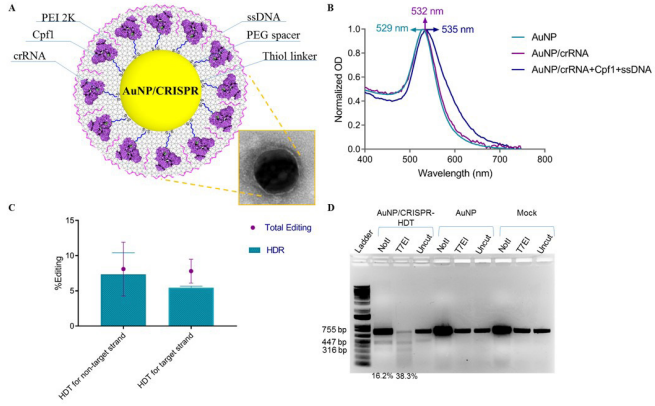

Figure 1. Characterization and gene editing efficiency of AuNP/CRISPR nanoformulations. A) Design of the AuNP/CRISPR nanoformulation. Inset shows the CRISPR coating on the surface of AuNPs. **B)** Respective localized surface plasmon resonance shifts representative of successful conjugation of components after each addition step. **C)** TIDE assay results showing total editing and HDR efficiency with templates designed for the target or non-target DNA strand. **D)** Restriction enzyme digestion showing the canonical cut bands after digestion with NotI and T7EI enzymes. HDT: homology directed repair template

378. Kidney-Directed Hydrodynamic Injection of *Slc3a1 piggyBac* Transposon Lowers Urinary Cystine in a Mouse Model of Cystinuria Type I

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Cystinuria type I is a disorder of renal amino acid transport, resulting in recurrent nephrolithiasis and significant morbidity caused by mutation of the *SLC3A1* gene. It is one of the most common autosomal recessive genetic disorders in humans with an incidence in the United States of 1 in 15000. The *SLC3A1* gene product is the neutral and basic amino acid transporter protein rBAT, expressed primarily in the proximal tubule cells of the kidney. *Slc3a1* -/- knockout mice are a mouse model of cystinuria type I, having elevated basic amino acids in their urine, decreased basic amino acids in their blood, and development of cystine stones in their bladders during adulthood. Hydrodynamic renal pelvis injection permits introduction of naked plasmid DNA specifically into the kidney, with expression observed mostly in the interstitial cells, tubules, and collecting duct. *piggyBac* transposons have been shown to have a high rate of integration into the genomes of mammalian cells, including in the liver, kidney, and lungs of mice following *in vivo* gene delivery. We created a *piggyBac* transposon expressing the *Slc3a1* gene product rBAT from the elongation factor 1 alpha promoter called pT-EF1a-*Slc3a1*. We confirmed expression of rBAT from this construct by Western blot analysis of transfected mouse embryonic fibroblast NIH 3T3 cell lysates. Following hydrodynamic renal pelvis injection, an increase in rBAT protein expression could be observed by immunofluorescent staining in knockout *Slc3a1* -/- mice. Delivery of the pT-EF1a-*Slc3a1* plasmid together with a plasmid expressing the hyperactive *piggyBac* transposase to adult *Slc3a1* -/- mice resulted in significantly reduced cystine levels in the urine at 4, 5, and 14 weeks following gene delivery as compared to mice receiving the hydrodynamic renal pelvis injection of buffer alone. Urine cystine levels were reduced by up to 70% in the group of mice receiving the transposon as compared with sham-treated animals. Although urinary cystine levels showed a promising improvement, analysis of cystine stones over time by x-ray revealed that the timecourse of stone formation was not significantly affected in the mice receiving pT-EF1a-*Slc3a1* and hyperactive *piggyBac* transposase as compared to sham controls. Our results demonstrate that kidney-directed injection of transposon DNA can mediate a biologic effect *in vivo* such as alleviation of cystinuria in a mouse model of this human disease. Future directions will focus on improving the efficiency of gene delivery to further decrease cystine levels in the urine and developing new methods to improve transfection efficiency of the proximal tubule *in vivo*.

379. Amphiphilic Triblock Copolymer Promotes Delivery of a Mini-Dystrophin Plasmid DNA in Skeletal Muscles of *Mdx* Rodents Following Hydrodynamic Limb Vein Injection

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Inherited myopathies are characterized by a progressive muscular weakness, which greatly affects the quality of life of patients, leading to a poor prognosis in their life expectancy. To date, no cure exists and treatments are only symptomatic. Gene therapy remains a promising approach to treat such disorders. Viral vectors like adeno-associated virus (AAV) can be very efficient for the transduction of skeletal muscles and some are currently tested in clinical trials. However, these vectors still also display some drawbacks, like possible immune response induction and large-scale production difficulties. Contrary to these vectors, synthetic delivery systems are characterized by a full-control of their manufacturing process and an in-depth characterization of their chemical structures and supramolecular assembly. Furthermore, they do not induce any immune response, thus making them suitable for repeated delivery required for such chronic pathologies. Their efficiency is still clinically insufficient and has to be improved. Beside this, the administration method is also a critical parameter. It has to be both safe and efficient while targeting the right tissue. Hydrodynamic limb vein (HLV) injection is a method that allows to treat at once the whole musculature of a given limb. Following this method, naked plasmid DNA (pDNA) can be transfected with some efficiency. Classically, to increase the yield of gene transfer compared to naked DNA, cationic (lipid or polymer) systems can be used. For the muscle, in this context, these chemicals have been revealed deleterious. However, others class of compounds exist. In the present work, neutral amphiphilic triblock copolymers (NATC) were investigated to determine their ability to deliver a mini-dystrophin encoding pDNA in the skeletal muscles of *mdx* rodents following HLV injection. Animals were injected to compare naked pDNA versus NATC/pDNA combinations, following simple or multiple HLV administrations. In each case, treatment was well tolerated. Muscles of the hind limb were analyzed by immunohistology. Interestingly, after repeated injections of pDNA mixed with a NATC, the global number of mini-dystrophin positive myofibers could be increased, and in certain muscles, more than 20% of fibers was transfected. We continue to optimize this approach in view to reach 40% of transfected myofibers from which a significant clinical benefit in patient should be obtained.

380. Rescue of Coat Color Phenotypes in Mutant Mice Using the Gonad Method

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Point mutations or retrotransposon insertions are quite common in many human genetic diseases. Disease phenotypes are expected to be rescued by correction of these disease-causing mutations by genome editing technology. It has long been known that some coat color phenotypes in mutant mice are caused by such genetic mutations. Here, we wonder whether Genome-editing via Oviductal Nucleic Acids Delivery (GONAD) technology, which does not require isolation of zygotes, nor *ex vivo* handling and subsequent transfer to recipient females to generate genome-edited mice, can be applied to correct these mutations. We chose the point mutation in the *Tyrosinase* (*Tyr*) gene as an example. This mutation changes cysteine to serine at amino acid 103 of the tyrosinase protein, leading to reduced activity in albino MCH(ICR) mice. If this mutation is corrected, rescued phenotype can be determined by observing eye pigmentation or coat color changing. GONAD was performed in 13 pregnant MCH(ICR) females. 36 individuals from a total of 74 offspring derived from these females exhibited the expected pigmentation phenotype with a percentage of 49%. We next tested whether GONAD method could be used to generate mice with large genomic deletions by targeting a retrotransposon sequence present in the 1st intron of the *agouti* locus in C57BL/6 mice. A genomic region spanning 16.2 kb containing the retrotransposon sequence was targeted for deletion. The deletion of this region should result in a coat color change. GONAD was performed in eight pregnant C57BL/6 females. Of these, six pups were recovered and three of them (50%) exhibited large deletions in their *agouti* locus and showed wildtype coat color. These results have shown that GONAD can be used to rescue pigmentation defects by correction of a point mutation and elimination of a retrotransposon sequence. Our strategy can be applicable to human germline gene therapy to correct disease-causing mutations. Considering that human germline gene therapy is most likely to be inseparable with *ex vivo* handling of embryos, including an *in vitro* cell culture step that could cause epigenetic changes to gene expression and affect fetal development. Favorably, since GONAD does not require *ex vivo* handling or sacrifice of GONAD-treated females, it offers a highly promising approach to human germline gene therapy in the future.

381. Intramuscular Electroporation of DNA-Based Monoclonal Antibodies in Sheep: A Translational Model for Clinical Antibody Gene Transfer

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Introduction: *In vivo* antibody gene transfer is an appealing alternative to conventional monoclonal antibody (mAb) protein production and administration. Delivery of mAb-encoding sequences allows the site of administration, e.g. muscle, to produce the mAb of interest in a cost- and labor-effective manner for a prolonged period of time. Applied expression platforms, mostly in pre-clinical settings, include viral vectors, plasmid DNA (pDNA), and mRNA [1]. We recently reported proof of concept for intramuscular DNA-based antibody gene electrotransfer in mice, achieving prolonged mAb expression at therapeutically effective plasma concentrations [2]. Despite pre-clinical progress, antibody gene transfer struggles to bridge the gap to the clinic. To accelerate clinical implementation, there is a clear need for supportive data in more translational models. **Aim and methods:** This study aims to evaluate intramuscular antibody gene electrotransfer in a clinically relevant animal model. We used 40-60 kg sheep, a clinical electroporation protocol and device (Cliniporator®, IGEA Medical), and a panel of newly engineered fully ovine DNA-based mAbs. Plasma mAb and anti-drug antibodies (ADAs) were measured using in-house developed ELISAs. **Results:** Intramuscular electroporation of 4.8 mg pDNA, encoding an ovine anti-human CEA mAb (OVAC), led to peak plasma concentrations up to 1 µg/ml 2 weeks after administration. An ADA response against the variable mAb region led to loss of OVAC detection 4 weeks after gene transfer. pOVAC re-dosing failed to restore mAb titers. Two strategies were pursued to address this humoral immune response. First, we swapped the OVAC variable region for that of an ovine anti-human EGFR mAb (OVAE). Intramuscular administration of 4.8 mg pOVAE in sheep led to peak plasma mAb concentrations of 300-700 ng/ml, 3 weeks after administration. No ADAs were detected and pOVAE re-dosing boosted mAb plasma levels. Second, we set up an immune suppression protocol in sheep. Following gene transfer of OVAC and concomitant administration of a clinical immune suppressant, mAb plasma concentrations peaked at single-digit µg/ml 3-5 weeks later, and were maintained within that range for at least 4 months. No ADAs were detected throughout the ongoing follow-up, despite immune cell counts returning to baseline within 2 weeks after immune suppressant administration. In immune-deficient mice, OVAE and OVAC plasma concentrations peaked at 10 and 30 µg/ml following a 60 µg pDNA intramuscular electrotransfer, respectively. Concentrations up to 5 µg/ml were maintained for at least 1 year after administration, illustrating the potential duration of mAb expression if an immune response is avoided. **Conclusions:** Our proof-of-concept data in sheep highlight the feasibility, scalability and challenges of clinical intramuscular

antibody gene electrotransfer. To advance this approach, the sheep model can be used to assess the impact of different variables, e.g. administration, dosing and enhanced construct engineering, in a highly translational setting. The application of this animal model also goes beyond DNA-based antibody gene transfer, as other expression platforms (e.g. viral vectors and mRNA) or encoded biologicals can be evaluated for translational ability

References:

[1] Hollevoet K, et al. State of play and clinical prospects of antibody gene transfer. J Transl Med, 2017; 15: 131-150. [2] Hollevoet K, et al. Prolonged *in vivo* expression and anti-tumor response of DNA-based anti-HER2 antibodies. Oncotarget, accepted.

AAV Vectors II

382. In Vivo Hepatic Transfer and Transcription of AAV Producer Plasmid Contaminants

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Adeno-associated virus is the most widely used viral gene therapy vector. It is known that during AAV production, AAV particles containing contaminating sequences are packaged. It is reported that sequences from the production plasmids are more likely to be packaged into AAV than producer cell line chromosomal DNA. Previous work has shown that these contaminants can have a negative impact on expression. We sought to characterize AAV contaminants in detail and look at their effect in vivo. Using a 293T transfection system, we produced a high contaminant AAV model, using an oversized gene construct (~5.2kb). AAV particle analysis showed producer plasmid contamination at greatest levels in 3 regions: in the expression cassette plasmid; proximal to the 5' ITR, and the 3' ITR, and in the REP/CAP producer plasmid; upstream of the P5 promoter. A capsid disruption assay indicated that sequences were packaged inside the particle. Certain contaminant amplicons were found to be present at >15% of the transgene titer by qPCR. Contaminant DNA abundances were verified by deep sequencing of particles, and confirmed that whilst sequences close to these regions are in high abundance, sequences remote from ITRs and the P5 binding site were undetectable. Comparatively, in an AAV preparation in which the expression cassette is optimally sized, a lower abundance of plasmid backbone sequences was observed, although the pattern of incorporation remains consistent. We examined transfer and retention of contaminant sequences in vivo and found contaminant sequences present within the hepatocytes of C57BL/6 mice after AAV infection at both a short time post infection (1-2 weeks) and long term (4 months). Contaminant sequences were detectable in hepatocytes for both high and low contamination preps by qPCR, and the pattern of sequences matched the AAV prep particle analysis. DNA deep-seq of infected hepatocytes confirmed this phenotype. Previous literature had indicated that AAV contaminants were not transcriptionally active. However, we hypothesized that as the preps analyzed in this way had a low contaminant contribution to start with, activity may have been below threshold detection. RT-PCR and RNA-seq analysis of mouse hepatocytes infected with our high contaminant AAV showed abundant transcription of all plasmid backbone contaminant sequences present in mouse hepatocytes, at both short and long term timepoints. Notably, the full length Kanamycin resistance gene and its transcript were detected by PCR from infected mouse hepatocytes. However, kanamycin resistance protein was undetectable. This is the first demonstration that producer plasmid contaminants packaged into AAV can be transcribed by a host system. Interestingly, the highly incorporated sequence from the REP/CAP plasmid is upstream of a cryptic Rep binding element in the P5 promoter, which suggests an active Rep mediated process of contaminant incorporation. Whether contaminant sequences are translated, and whether this phenomenon is limited to hepatocytes or present in other cell types, remains to be determined. The levels of plasmid backbone contaminants in AAV have previously been inversely correlated with transgene expression. These findings open an area of investigation that may shed light on the relationship between contaminant presence and transgene expression in AAV, and reiterates the importance of high quality characterization of clinically relevant AAV products.

383. Characterizing a Novel Antibody Epitope on the AAV5 Capsid at Atomic Resolution for the Creation of Host Antibody Resistant Recombinant Gene Delivery Vectors

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Adeno-associated virus serotype 5 (AAV5) is being developed as a gene delivery vector for several diseases, including cystic fibrosis, hemophilia, and Huntington's disease, and has demonstrated successful transduction of liver, lung, skeletal muscle, and the central nervous system. One of the limitations of the AAVs for gene delivery is pre-existing neutralizing antibodies in the human population. While the seroprevalence of AAV5 is lower than other serotypes, low levels of pre-existing immunity still presents a significant challenge for vector effectiveness in gene delivery applications. Here we report the cryo-electron microscopy and image reconstructed (cryo-EM) structure of AAV5 in complex with newly generated monoclonal antibody HL2476 to a resolution of 3.1 Å. To our knowledge, this is the highest-resolution antibody complex structure determined by cryo-EM to date. Unlike the other available anti-AAV5 capsid antibodies, ADK5a and ADK5b, with epitopes surrounding the 5-fold channel of the AAV5 capsid, HL2476 binds to the 3-fold protrusions. In addition, to fully understand the capsid-antibody interactions, the heavy and light chains of HL2476 were sequenced and built into the cryo-EM density map along with the AAV5 VP structure. The high resolution of the complex

enabled the identification of AAV5 capsid residues, in previously defined variable regions VR-V, VR-VI, and VR-VIII, involved in the antibody interaction. To confirm this and the footprints for ADK5a and ADK5b, a comprehensive panel of site-directed mutants, guided by the structures of AAV5 complexed with ADK5a, ADK5b, and HL2476, were generated. Native dot blot analysis and transduction assays, in the absence/presence of the parental MAbs as well as individual human sera and IVIG, are used to test the variants arising from this study to characterize antibody escape phenotypes. These studies identify single or few footprint residues as determinants of these interactions and highlight hotspots for engineering host immune escape vectors.

384. Distribution and Transduction of Multiple rAAV Serotype/Mutant Vectors in the Non-Human Primate Brain after Intracisternal Injection by an AAV DNA/RNA Barcoding Library

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Widespread gene delivery throughout the central nervous system (CNS) remains a major challenge to successful treatment of many neurological diseases. Delivery of recombinant adeno-associated virus (rAAV) vectors into the cerebrospinal fluid (CSF) can achieve gene delivery to cells throughout the brain and spinal cord; therefore, it represents a promising approach to target the CNS. However, how the rAAV vectors administrated into CSF distribute and transduce the CNS has yet to be determined systematically. Here we show that a novel barcoding approach, AAV DNA/RNA Barcode-Seq, enables comprehensive assessment of both vector genome distribution and transgene expression patterns of various rAAV vectors in the CNS and careful selection of natural AAV serotypes or engineered AAV capsids for specific therapeutic requirements. In this study, we sought to determine distribution and transduction profiles of various AAV strains in cynomolgus monkey brain by AAV DNA/RNA Barcode-Seq when the vectors are directly delivered into CSF. For this purpose, a barcoded-AAV library containing 58 different AAV strains (serotypes, variants isolated from animals, and capsid-engineered mutants) was injected into CSF via the cisterna magna of two cynomolgus macaques (3kg, $4x10^{12}$ vg/kg), and the brain coronal sections with 6-mm thickness were made 6 weeks post-injection. The right hemisphere slices were used to collect samples from 12 specified brain regions, and the left hemisphere slices were diced into small cubes (approx. 110 cubes per hemisphere) to generate three-dimensional distribution and transduction maps of each AAV strain. Total DNA and RNA were then extracted from each sample and subjected to the AAV Barcode-Seq analysis to determine relative quantities of vector genome DNAs and RNA transcripts of the 58 different AAV strains. This analysis identified AAV strains that can deliver vector genome DNA to the brain broadly and more efficiently than AAV9 and those that can mediate more efficient transgene expression regionally or globally compared to AAV9. In addition, the comparison between the vector DNA and RNA data revealed previously unappreciated AAV strain-dependent differences in vector genome transcriptional activities. Moreover, vector DNA qPCR data of each brain cube sample corroborated the difficulty in gene delivery to basal ganglia by any AAV strains via cisterna magna injection. Thus, these observations will help the choice of rAAV vectors for targeting a specific area or broad gene delivery in CNS.

385. Novel Liver-Directed Adeno-Associated Viral Capsid Variants with Striking Transduction Efficiency in Murine and Human Hepatocytes

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Adeno-associated viral (AAV) vectors have emerged as the most successful platform in liver-directed gene therapy. However, the efficacy of rAAV vectors equipped with natural serotype capsids is hampered due to immunological and cellular barriers, thus high vector doses are needed for therapeutic effects. The failure of natural AAV serotypes to transduce human liver as efficiently as preclinical animal models requires development of novel AAV variants, which can overcome species-dependent barriers and enable low-dose AAV vector treatment. In this study, we demonstrate the successful high-throughput *in vivo* selection of liver-directed AAV capsid variants that overcome these challenges. Specifically, we identified two candidates, MLiv1 and MLiv3, derived from an AAV2 peptide display library consisting of capsids with random 7-mer peptide insertions at amino acid position 587. rMLiv capsid variants were produced with high-titer, suggesting that capsid engineering did not interfere with capsid assembly or with genome packaging. Strikingly, *in vitro* transduction efficiencies of primary human and murine hepatocytes with rMLiv capsid variants were superior when compared to rAAV8 or the parental rAAV2. When assayed *in vivo*, both variants showed a strong liver tropism that was accompanied by significantly reduced accumulation in common off-target tissues such as spleen or lung. Furthermore, the *in vivo* transduction efficiency of both variants significantly outperformed rAAV2, and one of the two variants (rMLiv3) reached transduction levels comparable to rAAV8.

In summary, we report on two novel AAV2-based liver-directed capsid variants, which simplify development of novel treatment strategies as species-specific barriers were overcome. Moreover, due to favorable hepatotropism and improved transduction efficiencies, these novel variants may represent a valuable advance in the field of liver-directed gene therapy.

386. Comparison of Early and Late Gestation In Utero Gene Therapy, in a Non-Human Primate Model

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Intro: Progression of in utero gene therapy (IUGT) for various monogenetic disease has improved over the past decades. However, clinical success has been limited by low efficiency of gene transfer and poor expression of transgene level. Methods: We have designed a non-human primate (NHP) model of IUGT by injecting scAAV-LP1-hFIX (AAV5 or AAV8 pseudotypes) into the fetus at 0.4G (1-10 x 10^{10} vg/fetus, n=6) or 0.9G (4 x 10^{12} vg/fetus, n=5) gestation. Delivered offspring were serially monitored for transgene level, immune expression level and liver transaminase level. Interval biopsies of liver and peripheral tissues were performed to monitor temporal vector distribution through qPCR. Livers were visually examined during laparotomies and histologically examined to ascertain any evidence of hepatotoxicity. Results: Our results revealed that early recipients demonstrated lower median levels than late recipients (15.62 vs. 49.02%, p=0.01) while early AAV5 recipients showed a much lower expression than late AAV5 recipients (2.06 vs. 11.51%, p<0.0001). Overall transgene expression in early AAV8 was similar to late AAV5 subjects. No difference was apparent in the growth velocities of early and late IUGT recipients. Early recipients of both AAV5 and AAV8 showed a mild anti-AAV humoral response with initial reactions approaching but not crossing the positive threshold for the first 150 days post-IUGT. In comparison late recipients showed a more robust positive humoral response for the first 700 days post-IUGT particularly with AAV5-IUGT. At the same postnatal time-points early recipients had 2-3 log-folds lower vector copy per number (VCN) than their late IUGT counterparts in all tissues apart from cerebellum, pancreas, adrenal gland, gonads and peripheral organs: fat, skeletal muscle, skin, umbilical cord. Conclusion: In order to achieve desired outcomes of in IUGT, factors like gestation at intervention, and efficacy of vector should be optimized.

387. Optimizing the Inactivating Sequence Used to Make Protease-Activatable Adeno-Associated Virus Vectors

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Adeno-associated viruses (AAV) are promising vectors for gene therapy due to their low immunogenicity and lack of pathogenicity. However, AAV's broad tropism may lead to undesirable off-target effects in gene delivery applications. We have recently developed a protease-activatable virus based on AAV, called the provector, that has switchable behavior to allow for gene delivery only in the presence of matrix metalloproteinases (MMPs), which are elevated in diseases such as ovarian cancer, heart disease, and Alzheimers'. To create the provector, a locking peptide is inserted into the virus capsid to ablate cell receptor binding. The provector is locked until cleaved by MMPs, which allows for targeted delivery of genetic cargo to MMP-rich microenvironments while reducing off-site effects. The peptide lock includes two components, 1) the tetra-aspartic acid inactivating sequence (IS) whose presumed role is to inhibit native receptor binding and 2) flanking enzyme recognition sites that are cleaved in the presence of MMPs. We aimed to elucidate the design rule for the peptide lock sequence to optimize MMP-responsive provector and to aid in the translation of this platform to other viral vectors and environments. It has been unclear not only if the length of the IS impacts provector function but also if the presence of IS is even necessary. To define the design rule for the IS, we created mutants based on AAV serotype 9 with the IS composed of 0 to 10 aspartic acid residues. Increasing the length of the IS decreased transduction in the locked 'OFF' state without affecting the infectivity of the virus in the unlocked 'ON' state. Notably, an IS of length 0 (i.e. equivalent to having no IS in the peptide lock) shows no difference in transduction ability before and after unlocking, suggesting inclusion of an IS is necessary for provector function. An IS of 10 aspartic acids displays a 27-fold increase in transduction once treated with MMPs - the best ON/OFF ratio achieved thus far in any provector design. Both IS 0 and 10 provectors, however, appear to protect their genomes from nuclease digestion slightly less effectively compared to the wild-type capsid, while medium-length IS provectors are as effective as the wild-type capsid. Thus, we find the optimal aspartic acid-based IS to be 4-8 amino acids in length, with too long or too short resulting in suboptimal properties. Altering the IS does not appear to affect the ability of MMP to recognize and cleave the enzyme recognition sequence. Collectively, we demonstrate that the inactivating sequence is essential for PAV locking and offer a rudimentary design rule for the PAV platform.

388. Production and Characterization of Infectious Snake AAV Vectors with a Cross-Packaged AAV2-ITR Genome

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The high prevalence of pre-existing immunity against common AAV serotypes in humans poses a significant challenge to clinical translation of AAV vector-mediated gene therapy. Snake AAV is a novel AAV strain that was isolated from royal python in 2004 and its 4432-nt-long full viral genome sequence has been determined, including the inverted terminal repeats (ITRs) of 154 bases in length. A phylogenetic analysis of the viral capsid VP and AAP proteins shows that Snake AAV is remote from the common AAV strains and its closest neighbor is the autonomously replicating goose parvovirus. In our attempt to produce infectious Snake AAV vectors that can deliver transgenes to target cells, we replaced the AAV2 VP1 ORF (735 aa) of the commercially available pAAV-RC2 helper plasmid with the Snake AAV VP1 ORF (726 aa coded by the AY349010 DNA sequence), making pAAV-RCsn. Although this VP1 ORF exchange strategy has been successfully utilized for many other AAV strains, allowing for production of pseudotyped AAV vectors in HEK293 cells, none of our attempts resulted in successful production of Snake AAV particles. Recently, successful production of an AAV2-GFP vector using Snake AAV-ITRs and Snake Rep proteins has been reported; however, there has been, to our knowledge, no report of successful production of AAV vectors pseudotyped with the Snake AAV capsid using AAV2-ITR genome and AAV2 Rep proteins in HEK293 cells. Here we report for the first time that infectious Snake AAV vectors can be produced in HEK293 cells using a standard adenovirus-free plasmid transfection system with a minor, but non-trivial modification, and show that Snake AAV vectors are resistant to IVIG (intravenous immunoglobulin)-mediated neutralization. Western blot and RT-PCR experiments indicated that the failure of the production of Snake AAV vectors using the pAAV-RCsn AAV helper plasmid in HEK293 cells is due to impaired protein translation from this helper plasmid. To overcome this problem, we established a novel system that expressed Snake AAV VP1 and VP3 proteins from separate codon-optimized ORFs, under the control of the CMV-IE enhancer-promoter, together with the AAV2 Rep and Snake AAP proteins. Snake AAV-CMV-GFP and Snake AAV-CMV-luciferase vectors produced with this system successfully transduced viper heart (VH2) cells in a vector dose-dependent manner. As expected, adenovirus 5 co-infection significantly enhanced ssAAV vector transduction in VH2 cells. A neutralizing antibody assay demonstrated that Snake AAV vector can partially escape from antibody-mediated neutralization in the presence of IVIG at a physiological concentration. Although improvements will be needed for satisfactory transduction in human cells, our study indicates that AAVs from cold-blooded animals, such as reptiles, provide a potential AAV vector platform exhibiting a low prevalence of pre-existing immunity in humans.

389. Intrauterine Gene Transfer at Early Gestation with Safe Postnatal Vector Re-Administration: A Therapeutic Strategy for Early-Onset Congenital Disease

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Early intrauterine correction of genetic diseases may prevent the burden of pathogenesis and young recipients with minimal tissue damage respond better to gene transfer. There is concern of genome toxicity and other long-term adverse outcomes due to the anticipated vulnerability of the immature fetal genome. Here we describe the long-term outcomes of early gestation IUGT at 0.4G, using therapeutic scAAV8 and scAAV5 vectors expressing coagulation factors IX and X in an established non-human primate (NHP) model. AAV-FIX recipients (n=8) received $1x10^{12}$-$1x10^{13}$vg and AAV-FX recipients (n=9) received $1x10^{13}$vg at 0.3-0.5G, or $4x10^{12}$-$4x10^{13}$vg/kg by birth-weight. Mean AAV5-hFIX expression was 5.6±4.1%, significantly lower than mean levels among AAV8-hFIX infants at 17.7±5.7% ($p<0.0001$) over ~51 months and up to a 10-fold weight gain. Among hFX recipients, mean expression was similar between the two serotypes (AAV8 12.1% vs 12.0% AAV5, $p=1.0$) and was maintained over 35 months. Low persistent anti-AAV IgG levels were detectable from 0.6G and throughout the surveillance period, marginally higher among AAV8 recipients. Three AAV5 recipients were postnatally challenged due to hFIX expression <1%; with a dose of $2x10^{11}$vg/kg. All demonstrated improved peak expression ranging from 2.2-20.3%. One animal showed transient neutralising antibody production while the other two demonstrated activated CD4 and CD8 T cells. Only one animal showed significant increase in hepatocyte vector copy numbers (VCN) post-challenge. There was no serotype-dependent difference in organ VCN which was generally low and decreased with age. Stable transgene expression and very low VCN contributed to a transduction efficacy of 129.7μg/mL per vector copy of AAV8 and 806.7 μg/mL per vector copy of AAV5 by 36 months regardless of transgene expressed. There was no clinical evidence of hepatotoxicity despite mild biochemical transaminitis. Thus, early IUGT produces dose-dependent transgene expressions which may improve with postnatal challenge, limited by possible immune clearance by neutralising antibodies and activated T cells. Though it is generally believed that fetuses are immune-naïve, our data clearly shows that IUGT subjects are capable of mounting immune responses to AAV and retaining immune memory upon reintroduction of AAV. Despite this, early IUGT and postnatal challenges can be performed safely with the expectation of clinical benefit.

390. Genetic Modification of Human Bone Marrow Aspirates via Delivery of Raav Vectors Coated on Pnass-Grafted Poly(ε-Caprolactone) Scaffolds

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Introduction: Articular cartilage supports joint loading and motion but has a limited ability for self-healing. Gene transfer using the clinically relevant recombinant adeno-associated viral (rAAV) vectors is a powerful tool to enhance cartilage repair. Here, we tested the ability of various poly(sodium styrene sulfonate) grafted poly(ε-caprolactone) scaffolds to deliver rAAV to human bone marrow aspirates, a potential source of reparative cells for cartilage repair. ***Methods:*** The scaffolds were fabricated using a spin-coating method. For grafting the scaffold films with poly(sodium styrene sulfonate) (pNaSS), the films were suspended under stirring into distilled water at room temperature. The following conditions were tested: no coating, low coating (1.11 x 10-6 mol/g pNaSS), and high coating (1.30 x 10-5 mol/g pNaSS). rAAV vectors were packaged, purified, and titrated as previously described. rAAV-RFP carries the Discosoma sp. red fluorescent protein gene (RFP) and rAAV-lacZ the E. coli β-galactosidase (β-gal) gene (lacZ), both controlled by the CMV-IE promoter/enhancer. Vectors were labeled with Cy3 as described. Immobilization of rAAV on the scaffolds was performed by adding the vectors (40 µl) with 0.002% poly-L-lysine. rAAV release from the scaffolds was measured by the AAV Titration ELISA. Bone marrow aspirates were obtained from the distal femurs of donors undergoing total knee arthroplasty (n = 10). Aspirates (150 µl) were placed on rAAV-coated scaffolds with a mixture of fibrinogen (17 mg/ml)/thrombin (5 U/ml) (Baxter). The constructs were maintained in DMEM, 10% fetal bovine serum, 100 U/ml penicillin and streptomycin at 37°C for up to 14 days. Transgene expression was monitored by detection of live fluorescence and by X-Gal staining. ***Results:*** Successful immobilization of rAAV on the scaffolds (Fig. 1) was confirmed by detection of a fluorescent signal from Cy3-labeled vectors coated on the various pNaSS-grafted PCL scaffolds compared with scaffolds coated with unlabeled vectors (Fig. 2A). No clear difference were noted regarding the ability of the three types of scaffolds to incorporate rAAV. Remarkably, the various rAAV-coated scaffolds were capable of releasing the vectors over time, especially the low and highly coated pNaSS constructs (Fig. 2B). Effective scaffold-mediated rAAV gene transfer was achieved in human bone marrow aspirates over time as evidenced by detection of a strong fluorescent signal upon rAAV-RFP delivery versus control conditions (free vector application, uncoated scaffolds), regardless of the type of scaffold employed (Fig. 3A). Similar results were observed when the scaffolds were coated with rAAV-lacZ, revealing intense lacZ expression via X-Gal staining compared with control treatments (Fig. 3B). ***Conclusions:*** Effective modification of human bone marrow aspirates can be achieved by delivery of rAAV vectors from pNaSS-grafted PCL scaffolds. may provide effective systems to enhance cartilage repair in patients affected with focal tissue lesions.

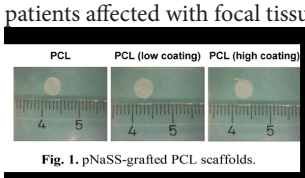

Fig. 1. pNaSS-grafted PCL scaffolds.

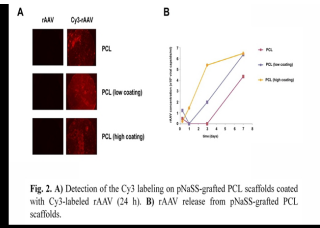

Fig. 2. A) Detection of the Cy3 labeling on pNaSS-grafted PCL scaffolds coated with Cy3-labeled rAAV (24 h). **B)** rAAV release from pNaSS-grafted PCL scaffolds.

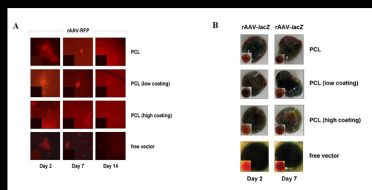

Fig. 3. A) RFP expression in human bone marrow aspirates incubated with rAAV-RFP-coated pNaSS-grafted PCL scaffolds (insets: uncoated scaffolds). **B)** *lacZ* expression in human bone marrow aspirates incubated with rAAV-lacZ-coated pNaSS-grafted PCL scaffolds (insets: uncoated scaffolds).

391. Persistence of rAAV in Peripheral Blood Cells and Hematopoietic Progenitors Upon Systemic Delivery

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Wild-type AAV have been shown to persist in healthy donors within circulating $CD34^+$ and peripheral blood mononuclear cells (PBMC). Although different preclinical studies revealed the ability of recombinant (r)AAV to transduce peripheral blood and stem populations, the eventual vector persistence within these compartments still remains poorly explored. In a clinical trial employing a rAAV-2/5 to deliver the defective enzyme for the treatment of acute intermittent porphyria, vector particles were cleared out from serum within 4-8 weeks after systemic delivery. However, vector genomes were detected in 6/8 patients by quantitative (q)PCR up to 12 weeks post-administration in peripheral blood mononuclear cells (PBMC) and long-term persistence was further confirmed in 2/3 patients 2 years after vector injection. Vector copy numbers within PBMCs decreased overtime resulting, on average, in $2.82x10^{-3}$ and $4.26x10^{-4}$ vector genomes per cell at 12 weeks and 2 years timepoints, respectively. Linear amplification-mediated (LAM)-PCR revealed that rAAV genomes persisted as both integrated and concatemeric structures with a slightly varying average integration frequency of $5.98x10^{-5}$

integration sites (IS) per cell. The integration profile retrieved showed genome-wide distributed IS in absence of integration hotspots, as well as no overtime persistence of particular integration events. In order to investigate whether the sustained rAAV persistence could arise from bone marrow transduction, qPCR and LAM-PCR were performed on non-human primate (NHP) PBMC and bone marrow mononuclear cells (BMNC) collected at different time points following intravenous rAAV-1 or rAAV-2/5 administration. In accordance with the data retrieved from patients, rAAV genomes were detected at both 6 and 12 months timepoints. LAM-PCR revealed that rAAV genomes persisted as both integrated and concatemeric (95%) structures showing a progressive reduction of concatemeric vector forms 1 year post-administration, which also correlated with the overtime 1.5-fold decrease in vector load. Intravenously delivered rAAV-1 and rAAV-2/5 were able to transduce BMNC, which exhibited a 1-2-fold higher VCN when compared to PBMC and an analog abundance of concatemeric structures. We retrieved 187 and 342 integration events from PBMC and BMNC, respectively, which exhibited a genome-wide distribution with no integration hotspots. Interestingly, rAAV genomes were detected in both $CD34^+$ and $CD34^-$ BMNC fractions by qPCR and an average 3.7-fold increased vector load was observed within the hematopoietic progenitor compartment. In summary, here we show that intravenously delivered rAAV exhibit long-term persistence in patients peripheral blood cells in absence of adverse events. Studies performed in the non-human primate model revealed that both rAAV-1 and rAAV-2/5 persist within hematopoietic progenitors with neither clonal outgrowth nor signs of malignant transformation. This indicates the safe integration profile of these vectors within this compartment thus further reinforcing rAAV safety for in vivo gene therapy.

392. Comparative Study of AAV for Gene Delivery after Subconjunctival Injection

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Purpose: AAV based gene therapy approaches in the posterior eye resulted in the first FDA approved gene therapy based drug for Leber congenital amaurosis. However, application of AAV vectorology to the anterior eye, in particular following subconjunctival injection, has been relatively unexplored. To determine the utility of this route for the treatment of various ocular disorders a survey of gene delivery via natural AAV serotypes was performed. Methods: AAV serotypes packaged with a self-complementary reporter were administered via subconjunctival injection to WT mice. Ocular examinations, including slit lamp analysis and tear sampling for vector shedding, were performed over 2 months. Quantitation of vector genomes and transgene-derived cDNA were correlated to whole globe immunofluorescence of the transgene product. To rationalize serotype transduction in mouse, and determine the conservation in human tissue, reported AAV cellular receptors were analyzed in aforementioned species by immunofluorescence. Result: Subconjunctivally delivered AAV vectors were well tolerated with vector shedding in tears out 2 weeks following the injection. AAV transduction was serotype-dependent in anterior segment tissues including the eye lid, conjunctiva, and cornea, as well as the periocular tissues including muscle and glands. Transgene expression in cornea was determined highest for AAV6 and AAV8, however their corneal distribution was remarkably different: AAV6 appeared restricted to the endothelium layer while AAV8 efficiently transduced the stromal layer. These observations contrast AAV2 which also resulted in corneal epithelial transduction. Reported AAV cellular receptors were not well correlated to vector transduction, however, were in some cases conserved among mouse and human ocular tissues. Conclusion: Subconjunctival administration of particular AAV serotypes may be a simple and safe targeted gene delivery route for ocular surface, corneal, and muscular diseases of eye.

393. *In Vivo* Selection of CNS-Specific Novel AAV Capsid Variants by Directed Evolution

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Adeno-associated viral (AAV) vectors are widely recognized as a very promising platform for gene therapy of neurological disorders. Directed evolution of AAV capsid shuffled libraries has been used as a powerful method to identify novel rAAV vectors with the desired tropism and transduction efficiency. In this study, we have successfully built an AAV capsid shuffled library with high complexity and diversity from 9 parental capsid serotypes (AAV2, 6, 7, 8, 9, rh8, rh10, rh39 and rh43). After 3 rounds of selection in non-human primate (NHP) with intrathecal (IT) injection, and in mice with *intrastriatal, intravenous (IV), and* IT injection, and 4 rounds of selection in human primary neurons and astrocytes *in vitro*, 24 lead capsid variants (>1% abundance) were selected. All selected capsid variants showed high complexity compared to parental capsids. Four of the 24 capsid variants have been characterized individually in mice using human frataxin-HA (hFXN-HA) as the transgene. At 4 weeks post-IT or IV injection, the mice were euthanized and CNS tissues and liver were collected. The expression of hFXN-HA mRNA and protein in the tissues was evaluated by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. Compared with the AAV9 vector, one of the variants showed higher expression of hFXN-HA at the mRNA and protein levels in the cortex and spinal cord, and two other variants showed stronger expression of hFXN-HA mRNA and protein in the cortex, brain stem, cerebellum and spinal cord. Administration of these two variants via IV injection also showed enhanced expression of hFXN-HA protein in the brain stem compared to AAV9. Moreover, all 4 capsid variants showed lower expression of hFXN-HA in the liver compared to AAV9. Taken together, the novel capsid variants selected from directed evolution of a capsid shuffled library show enhanced tropism and transduction efficiency in CNS tissues.

394. Identification of Novel Retinal Pericyte-Targeting RAAV Vectors through Directed Evolution

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Purpose: The ability to deliver genetic material to cells of the retinal vasculature may facilitate the development of long-term gene therapy treatments for sight threatening diseases, such as diabetic retinopathy or age-related macular degeneration. While recombinant adeno-associated virus (rAAV) vectors have been used extensively to target retinal neurons and glia, their utility for vascular gene transfer remains limited due to extremely poor transduction efficiency regardless of administration route. Herein, we employed a library screening approach to identify novel rAAV2-based capsid variants with improved ability to target retinal vascular pericytes following intravitreal delivery. **Methods:** Directed evolution was used to generate a combinatorial library of rAAV2 based capsid variants where mutations existed only in the variable regions of the capsid. Three rounds of *in vivo* selection were carried out to identify mutants with the greatest tropism for retinal pericytes. Briefly, 15-20 Cspg4-DsRed reporter mice per round received bilateral intravitreal injections of $1x10^{9-10}$ vector genomes. Retinal pericytes were harvested 6 days later by fluorescence-activated cell sorting (FACS) of enzymatically dissociated retinae followed by recovery of vector genomes (integrated and episomal) using column DNA purification. Following rounds one and two, the recovered capsid DNA sequences were amplified by PCR to prepare an enriched pericyte-targeting capsid library for the subsequent round of *in vivo* selection. After three rounds of selection, capsid DNA from retinal pericytes was amplified and inserted into pACG2-m56 and transformed, at which point 100-200 random clones were analyzed by next generation sequencing. **Results:** Following three rounds of screening, library complexity had reduced significantly, leading to the identification of seven novel capsid mutant variants with greatly enhanced ability to target retinal pericytes. A ubiquitously expressing GFP reporter construct was packaged into each of these novel mutant capsids and injected intravitreally into to Cspg4-DsRed reporter mice. A combination of confocal scanning laser ophthalmoscopy, post-mortem histology, and flow cytometry revealed that the novel capsid mutant vectors identified had substantially improved ability to target retinal pericytes than the unmodified rAAV2 serotype on which the library was based. **Conclusions:** Conducting multiple rounds of library screening under increasingly stringent conditions lead to a rapid decrease in library complexity and the isolation of several novel capsid mutant sequences with improved ability to target retinal pericytes. Despite the relative scarcity of retinal pericytes (>1,500 per eye) FACS followed by column-based DNA purification proved to be an effective technique for isolating target (i.e. red fluorescent) cells and recovering internalized vector genomes. The ability to target retinal pericytes using rAAV vector technology is highly encouraging for the development of a future gene therapy for diabetic retinopathy, a disease in which pericyte cell death precedes the development of sight threatening vascular complication, including proliferation and edema.

395. Using AAV Capsid Engineering and Spatial Transcriptomics to Dissect Circuit Repair in Parkinson's Disease

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Brain repair using embryonic stem cell (ESC) transplantation in Parkinson's disease has shown great promise as a future treatment option. However, little is known what directs maturation and circuit integration. In this study, we have transplanted human ESC-derived dopaminergic neurons into a 6-OHDA lesioned rat model and utilized retrograde infectivity of novel AAV's in combination with mono-synaptic rabies tracing, answer these outstanding questions. Establishment of the tracing starter population is generated through the Retrograde infectivity of the transplant using a Cre-inducible, FLP expressing AAV virus. As only the transplanted neurons express the Cre recombinase, this two-factor approach enable us to trace connectivity to only transplanted neurons which have managed to grow axons which regenerate the correct nigro-striatal circuitry. Using this novel AAV vector-induced tracing approach, followed by Spatial Transcriptomics mapping, we here present a more precise method to evaluate vector transport, vector function and mapping connectivity in the brain which provides significant advancement over current state-of-the-art. Using the ST technology, we have here deeply analyzed the maturation of hESC-derived DA neurons and compared them to both the intact midbrain DA-system and a reference single cell RNA-sequencing data set covering both human DA neuron development and midbrain neurons. With this approach, we show that the utilized differentiation protocol generates DA neurons which very closely mirrors a nigral gene expression with few genes originating from DA neurons from the ventral tegmental area. In addition, we show that nigral transplantation of identical hESC derived neurons receive distinctly different afferent input depending on the graft innervation pattern, suggesting a previously unknown directed circuit integration.

396. Capsid-Optimized AAV6 Vectors Result in Improved and Sustained Transduction in Murine Lungs without Genomic Integration

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Despite the progress made in AAV-based gene therapy targeting different organ systems, lung-targeted gene therapy using AAV vectors has not been effective, mostly due to poor transduction and un-sustained gene expression in airway epithelium. Furthermore, concerns over possible harmful insertional mutagenesis seen in

other cell types, particularly hepatocytes, raised a question about AAV safety. We have previously reported improved transduction of respiratory epithelial cells using novel capsid-optimized AAV6 vectors. In this study, we evaluate for long-term persistence of these optimized vectors in mouse lungs and possible integration of these vectors into the host genome. Lungs of ten-week-old C57BL/6 male mice were infected with a dose of $5x10^{10}$ vgs/animal with either AAV6-WT or capsid-optimized AAV6-Y705-731F+T492V. Vectors were delivered through intra-tracheal intubation and expressed firefly luciferase gene. Luciferase activity was measured weekly for the first-month post-infection and then monthly for a total period of 200 days (experiment #1) and 150 days (experiments #2) using a Xenogen IVIS Lumina System at same settings of FOV 12.5 and exposure time of 30 seconds. In both experiments, most animals maintained luciferase expression throughout. However, 2-3 weeks after infection, the expression dropped drastically (approximately 10-15-times from the maximum expression). Luciferase expression was significantly higher (1.5-2.5 times) in animals injected with AAV6-Y705-731F+T492V compared to AAV6-WT. At termination point of experiments, gDNA was extracted from the lungs and analyzed for AAV copy number by qPCR. On average, there were $1x10^4$-$2.5x10^4$ copies of AAV per 100 ug genomic DNA which corresponds to approximately 0.5-1.25 copies per mouse genome. No statistically significant difference was found between the two vectors. We then evaluated the possibility of integration of AAV expression cassette in the mouse genome in the same lung samples. The Sure Select XT Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library was used for gDNA library preparation. Barcoded DNA was pooled and sequenced (2 x 150 bp) on the Illumina MiSeq platform, yielding an average of 1.3 million paired reads per sample. DNA sequences were then mapped to the mouse reference genome (GRCm38) by using BWA (VN:0.7.15-r1140). The mean fold AAV-reference target coverage for AAV infected samples ranged between 5000x-23,000x versus 26x for the uninfected control samples. Overall, the fold enrichment of AAV vector target-specific sequences by the AAV capture bait ranged from 73,000-fold to 440,000-fold. The Samblaster (VN:0.1.24) was used to identify discordant read-pairs or split reads between AAV-vector and mouse genomic DNA. The fraction of discordant or split reads ranges from 0.88 to 1.72 per 1000 AAV-vector mapped reads (map quality >=30). We found that these reads were distributed broadly with no apparent systematic preference in any mouse chromosomal map location. We concluded that any inference of AAV integration is extremely weak and at best circumstantial. However, we cannot rule out the possibility of low-frequency AAV integration at levels below the sensitivity of sampling depth. In summary, our data suggest that AAV mediated long-term expression in lungs are visible and no risk of insertional mutagenesis with AAV integration was identified. **Acknowledgement:** This project is supported by funding from Katie Rose Cystic Fibrosis Research Fund at the University of Florida. We thank UF Toxicology Core for evaluation of AAV copy number, Genewiz for preparing DNA library, and UF ICBR BioInformatics Core for analysis of integration.

397. Mutagenic Analysis of an Adeno-Associated Viral Variant Simultaneously Inducing Immune Resistance and Efficient Gene Delivery

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Exploring safe and efficient viral vectors is a key prerequisite in pursuing the fundamentals of gene therapy approaches for the treatment of many incurable diseases. In addition to the capability of enhancing gene delivery efficiency in many problematic cells, the ability of circumventing neutralizing antibody (NAb) inactivation is of great importance that gene carriers must fulfill as therapeutic agents. Adeno-associated virus (AAV) is a highly versatile gene carrier that has been extensively utilized in gene therapy trials due to its lack of pathogenicity and its effective delivery characteristics. This study revealed that AAVr3.45, a genetically engineered variant based on AAV2, possesses two attractive properties as a gene carrier. First, a point mutation at 719 site substituted from valine to methionine renders AAVr3.45 more effectively bypassing immunoglobulin (IgG) neutralization apt to be utilized as *in vivo* vectors. Moreover, AAVr3.45 shows superior properties of evading antibody neutralization even exposed to the AAV-specific A20 monoclonal antibody set. It was assumed that the three-dimensional structure constructed by the surrounding peptides neighboring the 719 site or characteristic properties of side groups on the designated amino acids might serve as one of the primary epitopes that can be recognized by Nabs. Based on these hypothesis, the advantages of substitution to valine at the exposed site were revealed by the reverse mutation using site-directed mutagenesis. Second, peptide insertion (i.e. LATQVGQKTA) at the exposed heparan sulfate proteoglycan (HSPG) binding site dramatically increased the infectivity to the non-permissive cells. To interpret the roles of inserted peptide, one of special amino acids, lysine (K), was replaced with glutamic acid (E) and this variant shows dramatically decreased transduction efficiencies even without its exposure to the neutralizing antibodies. Finally, AAVr3.45 shows enhanced therapeutic efficacy by delivering pro-apoptotic genes to non-permissive breast cancer cells under the high titers of IgG conditions. A profound understanding of the AAV capsid structure will make contribution to the rational designing of multifunctional AAV vectors with selective organ targeting for the enhancement of therapeutic efficacy. The discoveries in this study will broaden the possible applications of AAV in gene therapy area by enhancing the knowledge of AAV virology for the further capsid designing.

398. Evaluation of Different AAV5 Gene Delivery Methods to the Central Nervous System and Application in Rats

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A major challenge for CNS-targeting gene therapies remains finding the appropriate delivery route to efficiently transduce the affected regions of the brain and/or spinal cord. We previously obtained good localized transduction of the striatum and frontal brain with AAV5 after intrastriatal administration. We now investigated 1) the ability of AAV5 to transduce different cell types within the nervous system and 2) biodistribution of AAV5 in the CNS upon administration in different structures of the brain in rats. Human iPSC were differentiated into frontal brain neurons, dopaminergic neurons, astrocytes and motor neurons. Immunohistochemistry with specific markers confirmed a successful differentiation rate. The cells were then transduced with AAV5-GFP and GFP expression was observed in all the different cell types. To explore transduction profiles *in vivo* following different routes of administration, rats were injected with AAV5-GFP in the striatum (2,5e10 gc), thalamus (2,5e10 gc) or cerebral ventricle (ICV, 5e11gc) and the vector distribution and GFP expression was evaluated 4 weeks post surgery. Intrastriatal injections resulted in a sustained transduction of the frontal and mid region of the brain with no leakage to peripheral organs. Delivery to the thalamus showed broad distribution throughout the whole brain, low transduction of the spinal cord and low leakage to peripheral organs. ICV injections resulted in the most widespread AAV5 distribution in the whole brain and spinal cord but the transduction efficiency was relatively low compared to striatal and thalamic delivery. Furthermore, high leakage of the vector to the liver, spleen and kidney was detected after ICV injection. GFP expression (mRNA) correlated well with vector distribution using all three delivery routes. We conclude that diseases with involvement of deep brain structures can be targeted by injection of AAV5 directly in the parenchyma of the striatum and thalamus. ICV administration of AAV5 could be more suitable for disorders where both brain and spinal cord needs to be targeted such as motor neuron diseases. Our *ex vivo* data in iPSC-neurons confirm AAV5 as a useful vector for CNS disorders efficiently transducing different neuronal cell types found in the brain and/or spinal cord. The current technology allows testing transduction efficacy and cell-specific functional effects in neuronal cells from patients. Thus, AAV5 has a broad neuronal tropism and has the potential to mediate gene transfer to the entire CNS dependent on the route of administration.

399. Identification of Novel AAV Capsids for Enhanced CNS Gene Transfer

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AAV9 is widely considered a "gold standard" capsid for central nervous system transduction. It has a broad transduction profile within the central nervous system. While administration of AAV9 via intravenous (IV) delivery can cross the blood-brain-barrier (BBB) and broadly transduce a wide range of cell types, AAV9 also strongly targets peripheral organs which could lead to transgene-related toxicity and/or enhanced immune responses. Alternatively, AAV9 can be delivered through intra-CSF administration (via cisterna magna or lumbar cistern) to provide broad CNS gene transfer with reduced peripheral organ biodistribution. Considering AAV9 as the best standard, we aimed to utilize a capsid DNA shuffling and directed evolution process to develop novel AAV capsids with 1) higher CNS gene transfer efficiency, 2) reduced peripheral organ biodistribution, and 3) an altered immunological presentation to avoid interaction with AAV9 antibodies. A total collection of 64 novel AAV capsid variants (referred as the AIM collection) was recovered after biopanning our library in mice following intrathecal (IT) administration. Analysis of these selected capsids is ongoing. To evaluate the biodistribution and cellular tropism of the recovered capsids, the vector variants were generated with a self-complementary GFP transgene. These were individually administered into wild type adult mice via lumbar IT or tail IV injection. Biodistribution analysis was preformed ten days post-injection by qPCR on the brain, spinal cord, dorsal root ganglia (DRG), sciatic nerve, and major peripheral organs. For capsids administered via the lumbar IT route, so far we report 10 variants that have transduction efficiency equivalent or greater than AAV9 in the brain and spinal cord, combine with reduced biodistribution to the liver. One of the capsid variants had CNS biodistribution about 5-fold higher than AAV9, and reduced peripheral biodistribution. Several of the capsid variants had somewhat specialized transduction patterns that were noted, relative to AAV9. This includes one variant with >80-fold higher efficiency to the sciatic nerve, along with a second variant that had >20-fold higher DRG biodistribution with minimal distribution to the brain. To examine the ability of the IT-selected variants to cross the BBB, we administered the capsids via tail IV injection in adult mice. While most variants did not traverse the BBB, one capsid had a brain biodistribution pattern equivalent to AAV9. Interestingly, some of the capsids showed peripheral organ tropisms that we were not selecting for, such as enhanced kidney biodistribution (relative to AAV9). Based on amino acid alignments, we would predict that these capsids would have low or absent cross-reactivity to antibodies specific for AAV9. Thus, there is potential for these capsids to be used to dose (or re-dose) patients seropositive to AAV9, or to be independent first-line vectors for CNS gene transfer. Future studies include assessing these candidate vectors in non-human primates and further screening of the AIM collection.

400. Lot-to-Lot Variation in Intrathecal Self-Complementary Adeno-Associated Virus Serotype 9 (scAAV9) Transduction

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Introduction: In comparison to small molecule drugs, viral vectors are complex biologics that pose unique challenges as they move towards the clinic. Impurities, such as empty or partially filled virions and virions packaging host cell DNA, are difficult to avoid completely, and the stoichiometry of the capsid subunits can currently only be measured in bulk. Anecdotes shared between investigators and the literature suggest that there is lab-to-lab variability in the transduction profiles of these vectors. For instance, Foust *et al.* (2009) found that intravascular scAAV9 primarily transduced astrocytes in adult mice, while Gray *et al.* (2011) found a predominance of neuronal transduction. Many things could account for these differences, such as dose, promoter choice, and the use of different animal strains. Vector production schemes could also play a role, as Goenawan *et al.* (2012) found that the timing of harvest affects the transduction profile of lentiviral vectors. Here we describe our own observations of lot-to-lot variation in scAAV9 preps. **Results:** An initial pilot study was conducted using a scAAV9-CMV-CBA-GFP vector produced at an academic vector core. In 70 day old rats, this lot showed widespread transduction of the spinal cord gray matter at all levels when given intrathecally. Based on these promising results, a proof-of-principle study was initiated using an scAAV9 vector expressing a therapeutic transgene and a new lot of the GFP vector as a control. In contrast to the pilot, GFP expression was almost exclusively limited to the dorsal root ganglia and absent from the gray matter. Anticipating that this might be an isolated problem, a third lot was tested, but it also showed negligible gray matter transduction. To determine how wide spread this issue might be, five additional scAAV9-GFP preparations were tested: two purchased directly from academic cores, two provided by industry collaborators, and one produced by a commercial vector core. Only two of the lots provided robust gray matter transduction in the spinal cord. Looking for a commonality among the lots that yielded gray matter transduction, we looked at differences in manufacturing (cesium banding vs. affinity chromatography) and formulation buffer. We also retitered the preps in a head-to-head comparison using quantitative PCR. Vector copy and formulation buffer did not correlate with potency of the vector, and an early hint that cesium banding might be beneficial did not hold up in a subsequent experiment. A GLP prep of scAAV9 expressing a therapeutic transgene also showed poor gray matter transduction, suggesting this issue is not limited to research-grade vector.We next considered that there might be differences in the contaminants present in each lot. In a pilot study, we performed charge detection mass spectrometry to evaluate the ratio of filled, partially filled, and empty capsids, as well as measure other high molecular weight contaminants. Two lots containing the same viral genome were evaluated, one that performed well *in vivo* and one that did not. A large fraction of both preparations consisted of partially filled capsids, suggesting that only half of the genome was packaged. The bad prep also showed a larger fraction of empty capsids and high molecular weight contaminants of unknown identity. **Conclusion:** Further study is needed to determine if these differences are directly related to the failure of the bad scAAV9 lot to transduce gray matter following intrathecal delivery. Our results highlight the need for better characterization of viral vector products to ensure consistency in potency as they move into the clinic.

401. Delayed Onset and Altered Biodistribution of a Non-Canonical AAV Entry Pathway

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Although many proteins and glycan factors have been proposed to play a potential role in AAV entry of specific serotypes, only recently an AAV entry receptor, AAVR was shown to act across a multitude of AAVs. We have identified a clade of primate AAVs evolutionarily related to AAV4 that do not require AAVR for entry or transduction. Further investigation revealed several additional serotypes that are able to undergo entry, although to a decreased extent, in the absence of AAVR both *in vitro* in cell culture and *in vivo* using an AAVR KO mouse model. Interestingly, cell binding assays using AAVR KO verses AAVR overexpression cells demonstrate no difference in binding of an AAVR-using serotype, AAV2, helping further define the role AAVR plays in the AAV attachment and entry process. We have determined that some previously categorized AAVR-dependent AAV serotypes are able to transduce an AAVR KO mouse, although with delayed onset of gene expression, and with an altered tissue expression pattern. In aggregate, our results demonstrate that AAVR usage is highly conserved amongst primate AAVs except for those in the AAV4 lineage, yet a non-AAVR pathway may be available to other serotypes, suggesting that some AAV capsids may have a multi-modal entry pathway.

402. Lyophilisation of AAV Gene Therapy Product

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Background: Biopharmaceuticals show varying levels of stability in aqueous solutions for short periods of time. Lyophilisation is a technique commonly used to improve the stability profile of biomolecules through the removal of water resulting in the increasingly restricted mobility of the reacting species. The gene therapy adeno-associated virus (AAV) subtype 8 containing Factor IX (FIX) (BAX335) was formulated in a new proprietary buffer and lyophilized. A stability study was established with the lyophilized material to determine its stability profile at the accelerated temperature of +5°C over a 10 month period. **Aim**: The goal of the study was to investigate the feasibility of lyophilizing the BAX335 drug product and to determine the possibility of extended storage/shipment of the drug product at +5°C. **Methods**:

The formulated BAX335 material was filled into glass vials. These were stoppered and freeze dried in an experimental lyophiliser. The lyo cycle used was not optimized but was based on glass transition and collapse temperature data collated with Differential Scanning Calorimetry (DSC) and Freeze Drying (FD) microscope, respectively. The cycle chosen was relatively long with very slow ramps so as not to stress the viral particles unduly. A stability study was established with the lyophilised material stored at +5°C for a period of 10 months. After defined time period, the AAV was reconstituted and tested. The stability samples were assayed with the appropriate analytical methods (pH, Appearance, total AAV ELISA, FIX-qPCR (vector genome), SEC (aggregates), WAX (%full AAV), *in vivo* and *in vitro* biopotency and %residual moisture) to determine the stability profile of the product over the course of the study **Results**: No significant change was seen with the viral drug product following the lyophilisation process. Some variation in the percent residual moisture was detected which was dependent on the position of the vials on the freeze-drying shelf. Over the course of the stability study, no significant change in the pH values and the appearance of the lyo cakes was detected. The % full AAV, aggregates and the total AAV count remained stable over the 10 month period. A recovery of 85% was obtained with the FIX-qPCR assay at the 10 month testing timepoint. The *in vitro* and *in vivo* biopotency assays did not show any significant loss in activity; all results were within the assay variation. **Conclusion**: This study has demonstrated the feasibility of lyophilisation of the AAV viral drug product in an appropriate formulation buffer. The freeze-dried product displayed an improved stability profile when stored at a temperature of +5°C. Additional studies are underway to optimize the lyophilisation program and to improve the lyo cake appearance through varying the sodium chloride concentration in the formulation buffer.

403. A Robust, Highly Sensitive Cell-Based Assay for Potency Measurement of the AAV-Vectorized Optogenetic Therapy, GS030-DP, Developed to Treat Retinitis Pigmentosa

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Potency is a critical quality attribute of biological product which is often complex to develop in an industrial compliant format for gene therapy product. However, this is an absolute regulatory requirement to comply with FDA's Current Good Manufacturing Practice (cGMP). GS030-Drug Product (DP) is an optogenetic AAV-vectorized gene-therapy developed to treat retinal degenerative diseases, such as retinitis pigmentosa, that will enter clinical stage in 2018. It encodes the algae red-shifted modified opsin, ChrimsonR-tdTomato, which essentially localizes to the cell membrane of transduced cells. Upon light stimulation at about 590 nm, ChrimsonR-tdTomato undergoes conformational change and opens, leading to a fast, proton- and cation-driven inward current, triggering plasma membrane depolarization. We describe here the development of a specific and highly sensitive potency assay assessing GS030-DP biological function i.e. light-induced depolarization of transduced cells. The bioassay uses an organic indicator dye and quencher combination which targets plasma membrane compartment of intact living cells and detects through fluorescence changes bidirectional membrane voltage gradient changes. The wide assay window and response linearity, together with sensitivity and no-wash homogeneous protocol, make this readout ideal to develop miniaturized tests in 384-well format readers, thus enabling simultaneous analysis of multiple assay conditions. Cells were thus seeded in 384-well plates, infected with increasing MOIs of GS030-DP and then analysed several days later with membrane potential sensitive dye. We used the FLIPR^{TETRA}, fluorescence plate imager, to deliver red light to excite GS030-DP-transduced cells and, simultaneously monitor the fluorescence changes of the voltage sensitive indicator. It shows, for the first time, the combination of a parallel fluorescent reader to an optogenetic drug screen. The potency of GS030-DP reference batch was determined under different assay conditions, such as cell number, infection timing and protocol, and assay temperature, in order to define the most suitable protocol to be applied for testing of clinical batches. Analysis of GS030-DP reference batch potency and signal dynamic range in large-scale tests (multiple infection rounds over multiple day and technical replicates) revealed a strong assay robustness and repeatability, as well as resistance to small deliberate variations of methodological procedure. Moreover, the sensitivity of the bioassay is considered high enough to detect small variation of functional potency of forthcoming GS030-DP batches. This robust potency assay will support the next steps of the GS030 gene therapy, especially for batch release and stability evaluation. This will also allow to comply with regulatory requirements in order to move to pivotal studies and ultimately to BLA/MA.

404. Stable Clone Generation for High-Titer AAV Production Using Single Cell Printing Technology, Combined with High-Resolution Imaging

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Multiple gene therapy programs have advanced in the past several years due to the introduction of new viral vectors, most notably the Adeno Associated Virus (AAV), where the first AAV based gene therapy was recently approved in the United States. As high yield vector production is needed to satisfy the demand of some of the targeted diseases, approaches to improve AAV production are necessary to ensure adequate supply. Often, parental and non-clonal cells, such as sf9 or HEK293 cells, are used in transient production systems, but the impact of the clonal heterogeneity on growth, and vector yield is unknown. In order to investigate this, subcloning of a suspension adapted cell line was performed using Single Cell Printing technology, which allows depositing a single cell per well of a micro-well plate, using real-time imaging combined with microfluidics capability, thus allowing for monoclonal colonies to be established. Additionally, high resolution imaging was used to confirm the clonality, and visualize the growth of the subclones. Differences in cell growth as well as production yield were observed amongst the subclones, resulting in a substantial improvement of viral vector yield. Comparison of product characteristics of the top subclones with the parental cell line is underway.

405. Biodistribution and Safety Study of AAV9 in Non-Human Primates

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In the framework of gene transfer studies for limb-girdle muscular dystrophy type 2A, a skeletal muscle disease that affects predominantly the proximal limb muscles and which is caused by genetic defects in calpain3 (*CAPN3*), we undertook a biodistribution and safety study in non-human primates (NHP). We designed rAAV vectors including the macaca fascicularis *CAPN3* coding sequence under the control of the hDesmin promoter. We first screened a group of fifty 2-3 years-old non-human primates for seronegativity to the AAV9 serotype and five 2/3-year-old male primates were included in the AAV study. We then evaluated the biodistribution and toxicity of our vectors after systemic delivery of a dose of 3e13vg/kg. As expected, the level of anti-AAV9 IgG increased dramatically following the injection, confirming the delivery of the AAV and the immunization of the primates for this serotype. Filter organs (liver, lymph nodes, spleen, lungs and kidneys) presented the highest level of Vector Copy Number (VCN) per diploid genome. The huge amount of VCN in liver was not associated to any toxicity or inflammation process. Samples from the nervous system and gonads were associated to the lowest level of detected VCN. The heart and skeletal muscles were in the middle range with a higher level in heart compared to skeletal muscles. Expression of the transgene was confirmed in heart and skeletal muscles. No noticeable variation of blood parameters was observed. In addition, several classical non-specific [creatine kinase, lactate dehydrogenase (LDH) and myoglobin] and specific cardiac biomarkers [Troponin T and N-terminus fragment of Brain Natriuretic Peptide (NT-proBNP)] were measured in the serum. Evaluation of the heart was also thoroughly examined at morphological and functional level by histology and echocardiography. The results show that no toxic or deleterious consequences were observed. In conclusion, upon delivery of 3e13vg/kg and after one month of expression, we showed that the single IV infusion did not lead to observable adverse effects or detectable toxicity in NHP.

406. Biodistribution and Shedding Studies of Adeno-Associated Virus 5 in C57BL/6 Mice by Quantitative PCR

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In the field of gene and cell therapy, biodistribution analysis is a key step in the evaluation of potential toxicity, as a part of safety assessment. Recently, quantitative PCR (qPCR) analysis has become the standard method for evaluating biodistribution of gene transfer vectors. However, there has been insufficient reference for studying and validating qPCR method. Thus, the present study aims to provide preclinical strategy of biodistribution and shedding studies of gene transfer vector by using qPCR technique. Among a number of different viral vector systems, adeno-associated virus (AAV) 5 was selected due to their lack of pathogenicity and ability to mediate long-term transgene expression. AAV5 ($2X10^9$ and $2X10^{11}$ copies/head) was inoculated by single I.M and I.V injection in female C57BL/6 mice (5 mice per group). qPCR method was validated according to Korea FDA guidance, and successfully performed in linearity, specificity, LOD, accuracy, and precision criteria. The results showed that although AAV5 was present in all organs tested (brain, infection site, liver, lung, spleen and ovary) over period of 29 days post-inoculation, the majority of AAV5 was detected in the liver and injection site. Ongoing investigations are focused on analyzing shedding and germline transmission of AAV5 by qPCR technique. In summary, our current study showed the preclinical biodistribution and shedding studies of AAV5 by qPCR technique. Further investigations into biodistribution, shedding and spreading, and germline transmission studies by qPCR technique may provide valuable insights into the future gene transfer therapy area.

407. Changes of Endogenous Gene Expression after VEGF Gene Therapy via AAV2 Double-Stranded Vectors: An In Vivo Study

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Background: We previously demonstrated that diabetic mouse (db/db) wound healing is significantly delayed with downregulated gene expression of multiple growth factors and their receptors including bFGF, PDGFb and VEGF. In this study, we aimed to investigate if adeno-associated double-stranded viral vector 2 (AAV2-ds) mediated VEGF gene therapy activates endogenous wound healing related genes. **Methods:** In 12 db/db mice, dorsal paired 8 mm-diameter wounds were created. 10^{11}viral particles of AAV2-ds-VEGF or AAV2-ds-GFP diluted in saline were injected intradermally into the wound edge and the wound bed, 6 mice per group. At day 15 and 21 post-wounding, six wounds from each group were formalin-fixed, paraffin-embedded (FFPE) and sectioned. Immunohistochemistry was used to verify the success of VEGF gene transfer. Total RNA was extracted from FFPE sections, wounds and edges separated. RT-qPCR was performed to examine gene expression of bFGF, EGF, IGF1, FN, MMP9, PDGFb, TGFb1, TIMP1, and VEGFa. **Results:** We verified significantly increased VEGF staining in AAV2-ds-VEGF treated wound granulation tissue at day 15. At day 15, the expression of PDGFb and TIMP1 genes was significantly increased ($p<0.05$ and $p<0.01$) in AAV2-ds-VEGF group compared to that in AAV2-ds-GFP group; the EGF gene expression was significantly higher in AAV2-ds-VEGF treated wounds than that in AAV2-ds-GFP treated wounds. At day 21, the expression of EGF gene in AAV2-ds-VEGF group was significantly decreased than that at day 15 ($p<0.05$). There was no significant difference in other studied genes. **Conclusions:** VEGF gene was successfully transduced by AAV2-ds vectors. The exogenous VEGF gene significantly increased endogenous genes specific to wound healing after AAV2-ds-VEGF therapy. Further optimization of AAV2-ds vector mediated VEGF gene therapy could improve the diabetic wound healing.

408. Alteration of AAV Capsid Lumenal Residues to Expand Vector Genome Packaging Capacity

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Adeno-associated virus (AAV) is a safe viral vector that has both evolved and been engineered to transduce a variety of clinically relevant tissues. However, several limitations exist for AAV including its relatively small DNA packaging size (4,700 nt). Existing serotypes have been over packaged with limited success and varying reproducibility. The consensus from the literature is that AAV can be over packaged by ~10%, but with a concomitant reduction in both viral titers and in vivo transduction. As a consequence, vector sequence and purification optimization are used to overcome the low titers to yield enough vector for clinical trials. We have taken a novel approach to increase the packaging limitations of AAV by focusing on the lumen of AAV where vector DNA interacts with the capsid. We have created a series of AAV capsid variants that alter the capsid lumenal charge using AAV-DJ as the scaffold. The variants add positively charged lysine, arginine, or histidine residues at lumenally exposed sites within the capsid. We hypothesize that the additional cationic charge density at the surface may induce DNA:capsid interaction altering or condensation, and leading to increased packaging capacity and/or stability in an over packaged state. We characterized a library of charge-altered variants to learn how lumenal alteration affects capsid stability and packaging capacity. We find that the vast majority of alterations actually allow the capsid to accommodate more mono-dispersed full-length packaged vector genomes above the wild type length of 4,700 nts. However, most of these variants transduce cells in vitro and in vivo poorly, because VP1 and VP2 are lost from the capsids. However, one variant maintains wild type VP1:VP2:VP3 ratios, while still retaining the ability to over package up to ~5,400 nts as a mono-dispersed vector genome. Despite the better apparent packaging capacity, the variant exhibits lower transduction compared to wild type AAV-DJ. We present data on biophysical characterization of the variant and strategies we have taken to overcome the apparent uncoupling between transduction and packaging. Approaches to make larger carrying capacity capsids while maintaining robust transduction are in progress.

409. Recombinant AAV Vector Design Influences its Genotoxic Potential

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Gene therapy utilizing recombinant adeno-associated virus (rAAV) has been shown to be a safe and effective treatment in mouse models of human disease. While it is less likely to promote genotoxicity than obligate integrating vectors such as retroviruses, studies have shown an increase in hepatocellular carcinoma (HCC) in mice treated with rAAV. The focus of this study is to determine what features of rAAV vectors could contribute to tumor promotion by recovering vector genome junctions from HCC in male C3H/HeJ mice. Liver tumors grow rapidly in these mice, increasing the sensitivity for detection of rAAV-associated oncogenic events. Half of the rAAV-treated mice underwent a 2/3 partial hepatectomy to induce rapid cell cycling. Different rAAV vectors were designed to examine how inclusion of introns, enhancer elements, and transgene expression influences tumorigenesis. Genomic DNA from tumors of mice treated with rAAV was enriched for vector sequence using a custom set of SureSelect RNA probes from Agilent. The enriched DNA was then sequenced on an Illumina HiSeq4000 sequencer. Vector integration sites were defined as any split read that aligned to both the vector genome and the mouse genome. Through this analysis, 102 unique integration sites were identified from 130 tumors analyzed. 70 of the 102 unique integration sites identified were either within or near known proto-oncogenes. All but 2 vector genomes found to be integrated in known proto-oncogenes contained only promoter elements. Overexpression of the associated proto-oncogene was hypothesized to contribute to cellular transformation in these tumors. The two integrations in which an intact vector was recovered had caused a truncation in EGFR that could lead to constitutive activation of the RAS pathway. Removing the SV40 intron from the scCBAnull (promoter only) and scCMV-GFP vector genomes had no quantitative effect on HCC incidence compared to vectors that contained an intron, or qualitative effect on the proto-oncogenes with which they were associated. However, no rAAV integration sites were recovered from any of the tumors of animals treated with the scCMV-GFP(Δintron). Removal of the enhancer element from the CBA promoter led to the greatest decrease in risk of developing HCC, and altered the locations from which vector integration sites were recovered. In general animals that were treated with a GFP vector and received a 2/3 hepatectomy had a lower risk of developing HCC than those that were treated and did not undergo hepatectomy. Moreover, hepatectomy status did not alter the genes from which integration sites were recovered. Taken together, truncated vectors are hypothesized to pose the greatest risk of causing cellular transformation, with integrated intact expression cassettes rarely being associated with HCC. Further, induction of synchronized cell division by 2/3 hepatectomy did not alter associated proto-oncogenes from which integration sites were recovered. This also led to a decrease in risk of developing HCC in the majority of animals treated with intact expression cassettes that more closely mimic clinical vectors, suggesting that a rapidly growing liver is not a risk factor for rAAV-associated tumorigenesis.

Cancer - Immunotherapy, Cancer Vaccines II

410. PD1-CD28 Switch Receptor Boosts Gene Modified T Cell Function through Evading Suppression from Tumor Microenvironment

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Safety and potency are two major requirements for an effective adoptive immunotherapy of cancers using genetically TCR engineered T cells. Current therapies of treating cancers using TCR engineered T cells, especially for solid tumors, are limited by poor T cell function and poor T cell persistence, majorly due to tumor microenvironment induced T hypofunction and exhaustion, as well as safety concerns due to mis-pairing of introduced TCR with endogenous TCR and using affinity enhanced TCRs that potentially cause un-predicted off target toxicities. In this study, we found high efficient multiplex gene disruption of both TRAC and TRBC in a wild type NY-ESO-1 TCR transferred T cells using CRSIPR/CAS9 gene editing, which led to profoundly improved antigen specific T cell functions both in vitro and in vivo of tumor mouse models. To further boost the in vivo anti-tumor activities of the NY-ESO-1 TCR transferred T cells, a switch receptor, comprising the truncated extracellular domain of PD1 and the transmembrane and cytoplasmic signaling domains of CD28 (PD1-CD28) was co-introduced into TRAC and TRBC disrupted NY-ESO-1 T cells. Treatment of mice bearing solid tumors with PD1-CD28 NY-ESO-1 T cells led to significant regression in tumor volume due to enhanced TIL infiltrate, decreased susceptibility to tumor-induced hypofunction, resistance of tumor derived suppression, such as from TGFβ, adenosine, hypoxia and Treg, compared to NY-ESO-1 T cells with TRAC/TRBC double disruption, or TRAC/TRBC/PD1 triple disruption. Moreover, RNA-seq showed massive gene expression profile changes in T cell activation, CD28 costimulatory signaling for PD1-CD28 NY-ESO-1 T cells with TRAC/TRBC double disruption. Furthermore, Additional disruption of Tim-3 for TRAC/TRBC double disrupted PD1-CD28 NY-ESO-1 T cells showed synergistic effect of controlling large, established solid tumors in mice. Taken together, our findings suggest that the application of PD1-CD28 to boost CRISPR/CAS9 gene edited TCR transduced T cell activity is efficacious against solid tumors via a variety of mechanisms, prompting clinical application of this potentially promising treatment modality.

411. Modulation of T Cell Antigen Processing and T Cell Signaling: a New Approach for the Generation of Allogeneic Cellular Therapy Products

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Background Chimeric antigen receptor T cell therapies using autologous T cells have recently been approved as therapy for relapsed or refractory diffuse large B cell lymphoma (DLBCL) and pediatric B cell acute lymphoblastic leukemia (B-ALL). However, widespread adoption and development of novel CAR T cell therapies are limited by the time, cost, and logistics associated with manufacturing an autologous gene-modified cell product. Conversely, an "off-the-shelf" cell product could substantially decrease manufacturing costs, clinical resource utilization and time-to-treatment for rapidly progressing patients. Efficient KO of the endogenous T cell receptor (TCR) is critical to abrogate the risk of graft vs host (GVH) from the allogeneic product given to an immunocompromised host. Conversely, to date, these allogeneic products have been expected to have short persistence due to recipient immune recognition of foreign antigen in the context of MHC. One way to overcome this T-cell mediated form of rejection is with HLA class-I knockouts (KO), but these are expected to be swiftly rejected by NK cells due to complete loss of HLA. We have taken a novel approach of using natural virus-derived inhibitors of Class I expression to achieve substantial downregulation of HLA Class I and antigen presentation, in combination with CRISPR/Cas9-mediated KO of the endogenous T cell receptor. ***Methods*** We identified several virus-derived modulators of HLA class I expression known to play important roles in immune-evasion. First, we constitutively transcribed viral HLA modulators and characterized HLA expression in primary human T cells, Jurkat and Raji cells. We identified several sgRNA sequences for efficient knock out of the TCR complex (TCR), and included these in tandem with viral HLA modulators. Cells were electroporated with Cas9 endonuclease mRNA for desired CRISPR mediated KO and evaluated by functional analysis including luciferase based killing assay, luminex cytokine profiling and flow cytometry. ***Results*** We found that expression of viral HLA modulators led to significant down regulation of class I molecules, resulting in decreased expression of HLA. Modulators from different viruses were differentially effective in B cells compared to T cells. Combined expression of multiple viral modulators did not further decrease the expression of HLA. When combined with lentiviral CAR constructs containing TCR sgRNA, we achieved efficient KO of endogenous TCR. TCR-deficient CAR-transduced cells retained on-target cytotoxicity and cytokine production. ***Conclusion*** Taken together these results show that viral modulators of antigen presentation efficiently lower class I expression without requiring gene editing of the Class I locus or B2 microglobulin. Furthermore, viral antigen modulators can be efficiently combined with TCR KO and lentiviral transduction to generate "off-the-shelf" CAR T cell products with reduced antigen presentation. These cell products maintain on-target antitumor effects, and may offer improved persistence by virtue of their reduced alloreactivity.

412. Novel T Cell Receptor Fusion Constructs TRuC™ Polypeptides Utilizing the Signaling Power of the Complete TCR Complex for Solid Tumor Therapy

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Cellular immunotherapy with genetically engineered T cells expressing tumor-targeting chimeric antigen receptors (CARs) or T-cell receptors (TCRs), has emerged as a promising treatment modality for a broad range of cancers. Despite success in hematological malignancies, CAR-T cells have shown poor efficacy in solid tumor indications. Here, we present a novel T cell engineering platform based on T Cell Receptor Fusion Constructs (TRuC™) proteins that have the potential to overcome many CAR-T and TCR-T shortfalls. Unlike CAR-T cells, the TRuC™ protein functionally integrates into the TCR complex thereby harnessing the full power of the entire TCR complex culminating in the reprograming of T cells to target tumor antigen in a non-human leukocyte antigen (HLA) restricted fashion. Here, we show preclinical evidence that underscores the efficacy of TRuC™-T cells (TC-210) recognizing tumor-cell associated mesothelin. Healthy donor (HD) derived TC-210 T cells showed robust anti-tumor activity *in vitro* and *in vivo* in mouse models of human lung adenocarcinoma and mesothelioma. When compared to a 41BBζ CAR-T expressing the same mesothelin binder, TC-210 T cells showed earlier activation, proliferation and long-term persistence at lower pro-inflammatory cytokine production. Transduction rates of TRuC™-T cells generated with T cells from ovarian and pancreatic cancer patients were similar to HD-derived TRuC-T cells. Likewise, patient-derived TRuC-T cells proliferated at a similar rate. In a mesothelioma xenograft model, ovarian cancer patient derived TRuC-T cells were able to clear tumors and persisted in blood and spleen for over 40 days until study end. Our findings demonstrate that mesothelin-specific TRuC-T cells made with T cells from cancer patients have the potential to eliminate tumors and present a promising approach for the treatment of solid tumors.

413. Lentivirus Integration Site Analysis Characterization of Ctl019

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Tisagenlecleucel (CTL019) is an autologous immunocellular cancer therapy representing the first immune gene therapy drug approved by the FDA on August 30th, 2017. Patient's T cells are reprogrammed with a transgene encoding a chimeric antigen receptor (CAR) that allows these T cells to precisely identify and eliminate CD19 expressing malignant and normal B cells in an antigen-dependent but MHC-independent manner. The gene transfer is accomplished via *ex vivo* transduction with a replication-deficient human lentiviral vector. Under the control of an EF1α promoter, the expressed transgene is comprised of a CD8α leader sequence, a murine single-chain antibody fragment (anti-CD19scFv), a CD8 hinge and transmembrane region and a 4-1BB (CD137) and CD3ζ (TCRζ) signalling domain. In summary, the expression of the transgene confers the transduced T cells with specificity for cells that express CD19. This study presents the investigation of genomic insertion sites (IS) from experimental lentiviral infections of T-cells from 12 cancer patients (6 ALL and 6 DLBCL) and 2 healthy controls, defining number and location of vector integrations, individual clonal contributions and integration preference in or near cancer genes. CTL019 IS studies were conducted in 12 GMP-produced cancer patient samples in triplicate analyses after measuring vector copy number (qPCR: 0.04 - 0.71) and transgene expression (flow cytometry: 3.7 - 42.3%). IS were recovered using shearing extension primer tag selection ligation-mediated PCR (S-EPTS/LM-PCR) followed by deep sequencing (MiSeq; Illumina) and analyzed using an adapted version of the Gene-IS pipeline. S-EPTS/LM-PCR randomly shears the genomic DNA by sonication, therefore eliminates restriction enzyme biases as well as reduces PCR biases resulting in a quantitative readout of individual clonal contributions. Informed Consent Form was obtained for all clinical samples analyzed as part of the clinical protocols CCTL019B2202 and CCTL019C2201, as well as from healthy volunteers obtained from Hemacare (Van Nuys, CA, United States). Read filtering tools correctly recognized about 1/3 of total sequences (11 of 30 Million) of which about 3.8 Million referred to unique raw IS and about 750,000 were found to be raw redundant. In total, we retrieved 33,324 IS with raw coverage bigger than one within the 14 samples. The unique IS number per sample ranged from 267 to 4,678, which is in line with the respective percentage of transduced cells. For all but one sample, the ten strongest clones represented less than 10 % of the read count indicating a polyclonal, not-clustered integration profile. Consequently, all samples showed polyclonal scores on traditional metrics (Richness, Shannon, Simpson and Eveness) or the recently developed PMD-Index. This finding was also supported by the 3 replicate analysis of the samples, where no IS were detected among the top clones in more than one replicate. Regarding IS targeting five genes previously associated with adverse events (*LMO2, CCND2, MECOM*) or clonal expansion (*IKZF1, HMGA2*), only *CCND2*, *MECOM* and *IKZF1* gene loci harbored 4, 1 and 5 IS, respectively. However, the relative abundance of the highest of them was far below 1 %. *LMO2* and *HMGA2* were not targeted at all by integrations within the 14 study samples. No substantial hotspots of vector IS were identified. The 12 clinical samples from the CTL019 project showed typical characteristics from conventional lentiviral IS in T-cells. There is no evidence for preferential integration near genes of concern or outgrowth of cells harboring IS of concern.

414. Characterization of Mesothelin-Specific T Cell Receptor Fusion Construct (TRuC™) Expressing T Cells for the Treatment of Solid Tumors

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Chimeric antigen receptor (CAR) T cell therapies have demonstrated impressive clinical efficacy in hematological malignancies, but these results have failed to translate in a solid tumor setting. Here, we describe a novel class of engineered T cells that integrate a T cell receptor fusion construct (TRuC™) into the natural T cell receptor complex, thereby reprogramming the specificity of the T cell to recognize tumor surface antigen in an human leukocyte antigen (HLA)-independent fashion. Unlike CAR-T cells, TRuC™-T cells utilize the entire TCR complex to deliver a diverse T cell activation cascade and have the potential to overcome limitations of CAR-T cells. We generated TRuC™-T cells (TC-210) that target mesothelin, neo-antigen expressed on an array of tumors. When tested for anti-tumor activity in a mesothelioma xenograft model, TC-210 cleared the tumors and protected mice from tumor re-challenge, indicating both potent and persisting anti-tumor activity. Interestingly, despite the potent efficacy, mouse serum cytokine analysis indicated that TC-210 T cell treated animals had lower levels of pro-inflammatory cytokines (IFN-γ, MCP-1, IL2, TNF-α) than animals treated with a 41BBζ CARs. To better understand the production of myeloid-derived cytokines, and the potential for cytokine release syndrome in a human-like setting, we compared the cytokine profiles of TC-210 and CAR- T cells in the presence of target cells and monocyte-derived antigen presenting cells. Relative to CAR-T cells, TC-210 T cells produced lower levels of IL-6, GM-CSF, TNF-α, IL-2 and IFN-γ . Taken together, our data demonstrate that mesothelin-targeting TRuC™-T cells are more potent and persist longer at lower levels of inflammatory cytokines than CAR-T cells underscoring the potential of TRuC™ T cells to treat mesothelin-positive solid tumors in clinic.

415. A Novel Non-Viral, Non-Integrative DNA Vector System for T-Cells Engineering

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Adoptive immunotherapy is one of the most encouraging therapeutic strategies for the treatment of a range of cancers. A particularly promising avenue of research is the functional introduction of Chimeric Antigen Receptors (CARs) into naive Human T-Cells for autologous-immunotherapy. Currently, the genetic engineering of these cells is achieved through the use of integrating vector systems such as lentiviruses or the sleeping beauty transposon system which present a potential risk of genotoxicity associated with their random genomic integration. We have invented a novel DNA Vector platform for the safe and efficient generation of genetically engineered T-Cells for Human Immunotherapy. This DNA vector system contains no viral components and comprises only clinically approved sequences, it does not integrate into the target cell's genome but it can replicate autonomously and extrachromosomally in the nuclei of dividing human primary cells. These DNA Vectors offer several advantages over currently used vector systems; they are not subject to commercial licenses, they are cheaper and easier to produce, and they can more quickly genetically modify human cells without the inherent risk of integrative mutagenesis. In preclinical experiments we have successfully generated genetically engineered human T-Cells that sustain the expression of a reporter gene for over a month at persistently high levels without decline. We have also successfully modified these cells with a range of transgenic CAR receptors against several known cancer cell epitopes and we have demonstrated their viability and capability in the targeted killing of these human cancer cells. We showed that CAR-T cells generated with our technology killed more efficiently target cells when compared to T Cells engineered with current state of art integrative lentivirus. The expression of functional CARs was detected over a period of two weeks of administration in culture and the anti-cancer activity of our DNA-CAR-T cells was evaluated *in vivo* using xenotransplanted cell lines in immunodeficient mice. We are currently performing analyses in order to determine the molecular behavior of the vector in the cells and its impact on cellular viability We believe that this novel DNA Vector system provides a unique and innovative approach to this exciting therapeutic strategy for cancer therapy. We estimate that this novel methodology will provide a simpler method of CAR T-cell manufacturing, resulting in a 10-fold reduction in the cost of the CART-product.

416. Abstract Withdrawn

417. PiggyBac Transposon-Mediated CAR-T Cells Targeting GM-CSF Receptors Prolong the Survival of Mice Bearing Human Acute Myeloid Leukemia

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Introduction Patients with relapsed/refractory acute myeloid leukemia (AML) have a poor prognosis. Although chimeric antigen receptor (CAR)-modified T cells redirected to AML-associated antigens have been tested in clinical trials, there have not been reports that have resulted in successful treatment of AML. We have previously developed

ligand-based CAR-T cells targeting GM-CSF receptors (GMR), and demonstrated that the CAR-T cells markedly inhibited the colony formation from CD34+ cells of juvenile myelomonocytic leukemia (J Hematol Oncol 2016). In the present study, we investigated the efficacy of GMR CAR-T cells for AML in vitro and in vivo using various AML cell lines. We also evaluated the effects of the CAR-T cells on normal blood cells. **Method** We modified the previously reported GMR CAR expression plasmid encoding GM-CSF linked to a hIgG1-CH2CH3, a CD28 endodomain, and a TCRζ chain so as not to have the hIgG1-CH2CH3 region. We used the piggyBac transposon system, according to our previous method (Mol Ther Methods Clin Dev in press), to non-virally engineer GMR CAR-T cells. We then co-cultured CAR-T cells with each of 5 AML cell lines as well as normal blood cells without cytokines at an E:T ratio of 1:1 or lower to evaluate their efficacy in vitro. Furthermore, we repeatedly co-cultured CAR-T cells with AML cells every 3-4 days at a 1:1 ratio to assess their sequential killing ability. As to investigate the in vivo efficacy of GMR CAR-T cells, overall survival of the AML cell-engrafted NSG mice treated with CAR-T cells, mock T cells or no T cells was recorded and analyzed. **Results** GMR CAR-T cells expressed 30-40% of CAR on the cell surface. Around 80% of GMR CAR-T cells were $CD45RA^{+}CD62L^{+}$ and $CD45RA^{+}CCR7^{+}$ displaying properties of naïve or stem cell-like memory. In coculture experiments, GMR CAR-T cells almost completely killed different subtypes of AML cells (acute myeloblastic leukemia with t(8;21), Kasumi-1; acute promyelocytic leukemia with t(15;17), HL-60; acute myelomonocytic leukemia, ShinM1; acute monocytic leukemia with t(9;11), THP-1; acute monocytic leukemia with t(4;11)/FLT3-ITD mutation, MV4-11) at an E:T ratio of 1:5. In sequential coculture, GMR CAR-T cells killed MV4-11 and THP-1 cells 3 or 4 times respectively at an E:T ratio of 1:1. The Kaplan-Meier survival curve displayed that GMR CAR-T cells improved relapse-free survival of MV4-11-engrafted mice by up to 60% for over 140 days (vs no T cells, p=0.0088; vs mock T cells, p=0.0066). All mice treated with no T cells or mock T cells died from leukemia progression within 70 days. GMR CAR-T cells did not attack B cells, T cells, NK cells, neutrophils, or monocytes. **Conclusions** GMR CAR-T cells are a promising candidate for the treatment of refractory/relapsed/FLT3-ITD+ AML. We are planning to conduct a clinical trial for AML using GMR CAR-T cells.

418. Controlled Expression of Interleukin-12 in the Tumor Microenvironment Using High-Capacity Adenoviral Vectors Eradicates Pancreatic Ductal Adenocarcinomas in a Murine Orthotopic Tumor Model

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We have developed High-Capacity adenoviral vectors (HC-Ad, also called Helper-Dependent or "gutless") equipped with a ubiquitous mifepristone-inducible expression system. Vectors were tested in a in a syngeneic orthotopic pancreatic cancer model in immune competent mice. Intratumoral injection of these vectors allowed local and controlled production of proteins, as demonstrated *in vivo* using luciferase as reporter. Genetic modification of the vector capsid to display RGD and poly-lysine motifs in the fiber knob reduced peritoneal spread. Different schedules of expression can be obtained after a single administration of vectors. When the immunostimulatory cytokine interleukin-12 (IL-12) was used as a therapeutic gene, the fiber-modified vector achieved the best tumor/serum ratio of IL-12 concentrations, which contributed to reduce toxicity of the treatment. Both wild type and fiber-modified vectors encoding IL-12 obtained a significant antitumor effect, with more than 50% of complete remissions. Cured animals were protected against a subcutaneous challenge with the same tumor cell line. We conclude that local, controlled IL-12 expression is safe and efficient against pancreatic cancer in a murine model.

419. CD8+ T Cells Express a Differential Transcriptome Signature in the Glioblastoma Tumor Microenvironment: Implications for Immunotherapy

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Background: Glioblastoma (GBM) is the most aggressive primary brain tumor in adults. These deadly cancers consist of a complex and diverse network of cell types, including tumor-infiltrating lymphocytes (TIL). This finding has led to the development of adoptive immunotherapy involving T lymphocytes for the treatment of CNS tumors. In order to better design such therapies it is important to understand the dynamics of TIL in GBM, specifically how the phenotype of TIL compare to the corresponding cells in the patient's own peripheral blood mononuclear cells (PBMC). **Material and Methods:** Using cell surface markers and intracellular cytokine staining we have been able to analyze and compare normal donor peripheral blood to GBM patients and verify which cell surface markers serve as surrogates for cytotoxic and T helper subsets, with an emphasis on Th17 cells. **Results:** On average GBM patients had comparable CD4/CD8 T cell ratios relative to normal donors. There was a trend towards and increase in Th1 (CD4+ IFN-gamma+), however this difference was not significant. Most striking was the increase in cytotoxic (CD8+ IFN-gamma+ cells) and IL-17 secreting cells (CD3+ CCR6+) in GBM patients relative to normal donors. This information was used to conduct an in-depth analysis of the TIL vs. PBMC phenotype. For 11 GBM patients cell surface markers validated using our staining of peripheral blood were used to enrich for and isolate 3 TIL subpopulations and compare their transcriptome to autologous peripheral blood subsets. Of the three subsets assessed (CD8+ T cells, CD4+ CCR6+ T cells and CD4+ CCR6- T cells), CD8+ tumor associated T cells (TIL) displayed the most dynamic expression profile when compared to peripheral blood counterparts, with

1587, 1 and 5 significantly differentially expressed RNA transcripts, respectively. The most common GOontology pathways altered in CD8+ TIL vs. PBMC included, extracellular space (secreted products), kinase activity, phosphorylation and the FoxO signaling pathway. Of the 1587 individual genes, 163 individual genes had a ≥2 fold change difference. As CD8+ T cells are thought to be the anti-tumor cytotoxic T cell workhorse cells, it is important to know how these genes could be affecting CD8+ T cell function. Among these genes were STAT3, LOC101929341 and MTHFD1L, which have been previously linked to inhibition of T cell recruitment to tumor sites, inhibited T cell proliferation of CD8+ autoreactive T cells and tumor specific CD8+ T cell hypofunction, respectively. Currently we are in the process of validated these genes using real-time PCR. We would like to use the information from this study to gain a better understanding of how the GBM tumor microenvironment could be impacting T cell function and its implications for cancer immunotherapy.

420. Synthetic DNA-Encoded Monoclonal Antibody Delivery (DMAb™) of Anti-CTLA4 Antibodies Induces Tumor Shrinkage In Vivo

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Antibody-based immune therapies targeting the T cell checkpoint inhibitors CTLA4 and PD1 have made incredible advances in cancer therapy. In particular, the combination of CTLA4 and PD1 blockade (using ipilimumab and nivolumab, respectively) was recently shown to be the most effective immune therapy for improving progression-free survival in advanced melanoma patients. However, this immune therapy combination strategy is extraordinarily expensive due to high manufacturing costs of monoclonal antibodies, with an estimated annual cost of almost one million dollars per patient. In this study, we focused on the development of a DNA-encoded monoclonal antibody (DMAb™) approach for delivery of anti-CTLA4 monoclonal antibodies *in vivo*. With this technology, formulated DMAb™ plasmids are injected into the muscle with electroporation, allowing for muscle cells to produce and secrete the DMAb™ for a prolonged period of time without the need for repeated administration. In proof of concept studies in mice, we show that delivery of a DMAb™ plasmid for a monoclonal antibody targeting mouse CTLA4 (clone 9D9) elicits high serum expression (peak expression of 7.9μg/mL in immune-competent C57Bl/6 mice with one injection of DNA). This anti-mouse CTLA4 DMAb™ is capable of inducing tumor regression in A/J mice that were implanted with the immunogenic Sa1N tumor cell line without repeated administration. Furthermore, this anti-mouse CTLA4 DMAb™ is capable of synergizing with a DNA vaccine targeting the tumor antigen TERT in slowing tumor growth for the non-immunogenic TC-1 tumor cell line. We also examined DNA-delivery of the anti-human CTLA4 antibodies ipilimumab and tremelimumab. For these anti-human CTLA4 DMAbs™, we achieved steady-state levels of approximately 75μg/mL and 50ug/mL for ipilimumab and tremelimumab respectively in mice, which is greater than the mean trough levels of ipilimumab achieved in patients (21.8μg/mL serum concentration for 3mg/kg dose). These anti-human CTLA4 DMAbs™ produced *in vivo* bind to human CTLA4 protein and induce T cell activation in a functional assay *ex vivo*. These results demonstrate the feasibility of delivering immune checkpoint blockade monoclonal antibodies using DNA for cancer immune therapy.

421. Electrochemotherapy in Combination with Interleukin-12 Gene Electrotransfer Generates Effective Anti-Cancer Immunological Responses in Murine Melanoma

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Background: Combining established therapies with recently discovered immunotherapies is a promising approach for treating malignant melanoma. Our research group has been studying intratumoral electrochemotherapy (a combination of a chemotherapeutic drug and electroporation), which is a well-established therapy in clinics across Europe [1]. Besides local release or exposition of damage-associated molecular patterns and neoantigens, electrochemotherapy with cisplatin, oxaliplatin or bleomycin induces immunogenic cell death [2,3]. Although local effectiveness of electrochemotherapy is up to 80% of local tumor control, no noticeable effects on distant non-treated metastases have been observed [1]. We hypothesized that gene electrotransfer of plasmid encoding for interleukin-12 (IL-12) as an adjuvant immunotherapy, compliments the responses seen with electrochemotherapy on a local and systemic level [4]. **Material and methods:** The combination therapy was tested using B16F10 malignant melanoma primary tumor model and a dual-flank melanoma model mimicking systemic disease. Treatment, consisting of intratumoral electrochemotherapy with cisplatin, oxaliplatin or bleomycin and peritumoral gene electrotransfer of IL-12, was performed when tumors reached 35 mm^3 in volume. Thereafter, tumor growth was followed and tumor and blood samples were collected for immunohistochemichal analysis of the tumor microenvironment and for the detection of IL-12. **Results:** The effect of combination therapies consisting of equally effective electrochemotherapy (low doses of cisplatin, oxaliplatin or bleomycin), combined with peritumoral gene electrotransfer of IL-12 was investigated. In comparison to either monotherapy which resulted only in a delayed growth of treated tumors, with the combination of electrochemotherapy and IL-12 up to 40 % of mice were cured. After the combination therapy, consisting of cisplatin in electrochemotherapy with an IL-12 boost, complete responses and a delayed tumor growth of distant non-treated tumor in the dual-flank melanoma model were observed. The induction of the immune response on a local and systemic level was observed; infiltration of granzyme B positive immune cells was detected in both, primary and distant tumors. **Conclusions:** We showed that peritumoral gene electrotransfer of IL-12 potentiates the effect of electrochemotherapy on local and systemic level. Our findings indicate a potential clinical application of the combination therapy for malignant melanoma treatment. **References:** 1. Yarmush ML, Golberg A, Serša G, Kotnik T, Miklavčič D. Electroporation-Based

Technologies for Medicine: Principles, Applications, and Challenges. Annu Rev Biomed Eng. 2014; 16:295-320 2. Calvet CY, Famin D, André FM, Mir LM. Electrochemotherapy with bleomycin induces hallmarks of immunogenic cell death in murine colon cancer cells. Oncoimmunology. 2014; 3: e28131 3. Ursic K, Kos S, Kamensek U, Cemazar M, Scancar J, Bucek S, et al. Comparable effectiveness and immunomodulatory actions of oxaliplatin and cisplatin in electrochemotherapy of murine melanoma. Bioelectrochemistry. 2018; 119:161-71 4. Sersa G, Teissie J, Cemazar M, Signori E, Kamensek U, Marshall G, Miklavcic D. Electrochemotherapy of tumors as in situ vaccination boosted by immunogene electrotransfer. Cancer Immunol Immunother. 2018; 64:1315-27

422. Placental Endothelial Cell Based Anti-Angiogenesis Immunotherapy: The Road to FDA Clearance of ValloVax

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Background: In recent years the most promising strides in the war on cancer have been made in the areas of cancer angiogenesis and cancer immunotherapy. To our knowledge, ValloVax is the first therapeutic cancer vaccine to be cleared by the FDA for clinical trials that leverages immunotherapy to evoke inhibition of angiogenesis. **Rationale:** The placental endothelium is known to possess numerous homologies with tumor endothelium in terms of proliferative status, growth factor receptors, and surface markers. ValloVax was created from isolated placental endothelial progenitor cells that are expanded ex vivo under conditions designed to maintain expression of tumor antigenic markers. **Preclinical Results:** ValloVax effectively inhibits tumor growth in models of breast cancer, melanoma, colorectal cancer, glioma, and lung cancer. Synergy has been demonstrated with classical checkpoint inhibitors, as well as various immune modulators. Animal studies demonstrate no adverse effects in acute and long term studies. **Clinical Trial:** The FDA cleared IND# 16296 for a Phase I/II trial assessing safety and signals of efficacy in 2 groups of 5 advanced lung cancer patients. Group 1: 25 million cells; and b) Group 2; 50 million cells of irradiated ValloVax subcutaneously into the abdominal area. Each injection will be in a volume of 2 ml. ValloVax administration will be performed weekly on days 0, 7, and 14 for a total of 3 injections. Immunological and imaging assessments will be performed at baseline, three months, and six months post treatment. Under a parallel Investigator Initiated trial 20 patients have been treated to date. Safety data and outcomes will be discussed.

423. Development of a Fast Screening Method for Chimeric Antigen Receptor Expression and Function

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Using chimeric antigen receptor (CAR) T cells to specifically target malignant cells is an exciting area of research that holds significant promise for treating a myriad of diseases. CAR T cells are redirected to target antigens via the expression of a single chain variable fragment (scFv), together with different components of the T-cell activation machinery. The commonly used process of screening new CARs in primary human T cells is often expensive, labor intensive and time consuming. Jurkat and HUT78 cells are two T cell lymphoma lines that have been utilized to dissect the components that make up T-cell signaling. Recently Jurkat cells have also been used to express CAR and the expression of an early activation marker, CD69 on Jurkat cells was reported upon target antigen exposure in vitro. Here we aim to investigate if using these cell lines to test the expression and functionality of CAR would be reflective of what is detected in primary human T cells. We transduced and generated Jurkat CAR T cell and HUT78 CAR T cell lines using lentiviruses encoding different versions of CARs targeting antigens expressed in glioblastoma, multiple myeloma, and B-cell lymphoma. The antigen specific activation and effector activity of these CARs in primary T cells have been previously studied. To determine the specificity of the CAR to an antigen, we co-cultured the CAR expressing cells with positive and negative antigen expressing lines. After 24 or 48 hours we measured activation markers including CD69 and 41BB (CD137) to indicate the activation levels of the CAR positive cell lines. We compared the observed expression pattern to that of the primary CAR T cells in addition to their functionality. We found that the expression of CAR on the surface of HUT78 cells more accurately represented that of primary human T cells. Furthermore, we showed that co-culture of CAR expressing HUT78 and antigen positive cells mimicked the pattern of activation seen in primary human T cells. One example is shown in Figure 1. This was not the case when we used CAR expressing Jurkat cells. Finally, we demonstratedthat 41BB (CD137) expression is a better predictor of activation levels and patterns when compared to CD69 in HUT78 CAR cells. In conclusion, using HUT78 cells as a model for screening CAR allowed us to closely predict the expression, specificity and functionality of the CAR in primary human T-cells. Using Jurkat cells can only indicate the presence of antigen driven activation. The validity of cell line based screening method has been reproducibly tested in different CARs with various tumor targets and it harbors a great potential to accelerate the CAR T development for a given target.

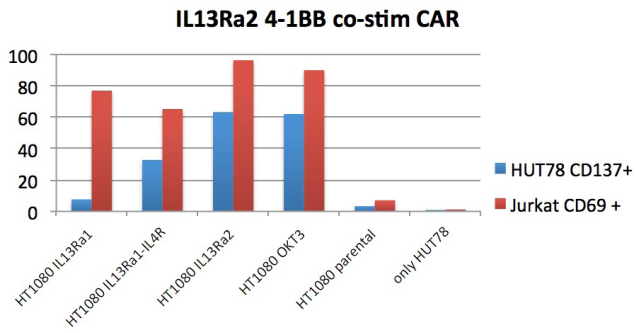

Figure 1: Antigen-dependent CD137 upregulation in HUT78CAR T cells recapitulated the activation pattern seen in primary CAR T cells. HUT78 and Jurkat cells transduced to express IL13Ra2 CAR were co-cultured with HT1080 cells expressing IL13Ra1, IL13Ra1-IL4R, IL13Ra2, or OKT3 (positive control for activation). After 48 hours of co-culture cell surface staining of CD69 and CD137 were determined. The pattern of activation in primary IL13Ra2 CART cells showed a high level of activation in response to IL13Ra2, a moderate response to IL13Ra1-IL4R, and a low response to IL13Ra1 (data not shown). A similar pattern was detected in the HUT78 CAR T cells using CD137 staining, but not the Jurkat CAR T cells using CD69 staining.

424. Valproic Acid Upregulates Expression of NKG2D Ligands & Increases Cytotoxicity of γδ T Cells against Acute Myeloid Leukemia

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Relapsed and refractory acute myeloid leukemia (AML) remains a critical clinical challenge, highlighting a need for safer and more targeted therapies for these patient populations. Gamma delta (γδ) T cells are an attractive candidate for adoptive cell therapy as they bridge the innate and adaptive immune systems and can stimulate cell lysis through both direct and indirect mechanisms. One of these cytotoxic mechanisms is through expression of the NKG2D (natural-killer group 2, member D) receptor and their interaction with NKG2D ligands such as MICA/B and UL-16 binding proteins (ULPB), which are over-expressed on cancer cells. Previous studies have shown that epigenetic modification using a histone deacetylase (HDAC) inhibitor, valproic acid (VPA), upregulates expression of NKG2D ligands on AML cell lines. Thus, our hypothesis was that AML cells treated with VPA would result in increased susceptibility to γδ T cell-mediated cytotoxicity via upregulation of NKG2D ligands. Our laboratory has previously developed a good manufacturing practice (GMP) compliant method to expand γδ T cells from peripheral blood using serum-free media in combination with bisphosphonates and cytokines. We treated three AML cell lines, MV4-11, Kasumi-1 and Nomo-1, and one chronic myeloid leukemia (CML) cell line, K562, with 1 mM VPA for 24 hours and performed flow cytometry to measure the change in NKG2D ligand expression levels between untreated control and VPA treated cells. VPA upregulated the NKG2D ligand expression in a cell line dependent manner, showing increased MICA/B expression in K562 cells, increased ULBP1 expression in MV4-11 cells, and elevated ULBP2/5/6 expression in MV4-11, Kasumi-1, and Nomo-1 cells. Median fluorescence intensity (MFI) for MICA/B expression was increased by 23.6 ± 7.45% in the treated K562 cells compared to untreated controls ($p<0.05$) and MFI for ULBP1 expression in MV4-11 cells was increased by 57.9 ± 12.4% compared to untreated controls ($p<0.05$). We found ULBP2/5/6 expression increased for the VPA treated Kasumi-1 and Nomo-1 cells compared to the untreated controls by 56.3 ± 2.58% and 58.1 ± 7.98%, respectively ($p<0.05$). γδ T cells were expanded in serum free media from peripheral blood mononuclear cells obtained from a healthy donor, resulting in an increase of the γδ T cell population from 1% to 80% ± 10% of the total cell population. For our cytotoxicity studies, day 15 *ex vivo* expanded γδ T cells were used as effector cells and MV4-11 AML cells were used as target cells. Cytotoxicity was measured by flow cytometry, which demonstrated a 67% increase in cytotoxicity at the 5:1 effector to target ratio compared to the untreated cells *($p<0.05$)*. Our data show that epigenetic modification of AML cells using the HDAC inhibitor VPA increases cytotoxicity capabilities of γδ T cells through upregulation of NKG2D ligands and can provide an option for adoptive cell therapy in patients with refractory or relapsed AML. Our future studies will involve testing AML cell lines and primary patient samples for increased NKG2D ligand expression using other epigenetic modifiers, including hypomethylating agents and other HDAC inhibitors, followed by testing the efficacy of combination therapy with γδ T cells in an *in vivo* AML murine xenotransplantation model.

425. Engineering T Cells and B Cells for Immunotherapy Using V(D)J Recombination

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T cell engineering for immunotherapy has shown clinical success, but large scale application is hindered by reliance on cumbersome *ex vivo* manipulations. In addition, B cell engineering has not shown therapeutic efficacy to date. Conversely, recombinant Adeno Associated vectors (rAAV) allow *in vivo* lymphocyte transduction but are seldom used for immunotherapy as they rarely integrate for stable expression in dividing cells. Here, we propose a novel immunotherapy approach -"VDJ targeting": targeting rAAV-delivered immune genes into the genome using V(D)J recombination in developing lymphocytes (Fig. 1). A promoterless receptor/Ab gene flanked by recognition signal sequences (RSS) is inserted into the endogenous locus by the recombination activating gene (RAG) complex during V(D)J recombination. In particular, in the T lineage, we target chimeric antigen receptor (CAR) or T cell receptor (TCR) genes into loci coding TCR chains and, in the B lineage, we target antibody (Ab) genes into loci coding Ab chains. Here, we demonstrate VDJ targeting in the B and T lineages in immortalized and inducibly differentiating cells as well as in in vitro differentiating primary lymphocytes and in fresh murine bone marrow. Next, we aim to demonstrate the therapeutic application of lymphocyte engineered by VDJ targeting. VDJ targeting may have several advantages over state of the art technologies: Only developing lymphocytes, expressing RAG, incorporate the receptor/Ab gene, which is subsequently expressed in potent naïve cells. Targeted T cells may express only the desired receptors, due to allelic exclusion, while targeted B cells may express the transgene as a B cell receptor, and upon activation undergo affinity maturation allowing potent immune

response, memory retention and diminished antigenic escape. VDJ targeting may allow safe, efficient and scalable engineering of B cells and T cells, both *in vivo* and *ex vivo*.

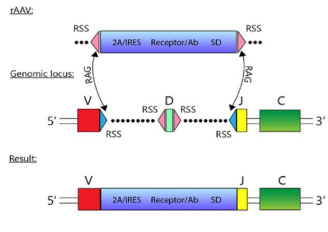

Figure 1: VDJ targeting: The rAAV vector encodes a receptor or Ab gene flanked by RSSs. Transduction of lymphocyte precursors, which express RAG, facilitates insertion of the receptor/Ab gene between gene segments. In the example presented, the transgene cassette is targeted between a V segment and a J segment, and the J recombination event may precede the V recombination event. The receptor/Ab gene is preceded by a sequence coding for a 2A peptide or an IRES to prevent protein fusions to preceding segments. A splice donor (SD) may be provided to allow use of the endogenous constant segments, upon integration and expression

426. ECAR-NK Cell Platform: Enhancing the CAR-Mediated NK Cell Cytotoxicity via Blockade of the CD47/SIRPa Axis

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Dramatic clinical responses were seen in the trials of adoptively transferred CAR T cells in hematologic malignancies. This success, however, could not be recapitulated in the context of solid tumors, likely due to the inefficient cell trafficking, hostile tumor microenvironment and tumor cell heterogeneity. New approaches are therefore needed to render CART cells more active, persistent and resistant to the inhibitory tumor microenvironment, as well as to move the CART cell therapy to the allogeneic/universal format. One option is to use allogeneic human NK cell lines as carriers of CARs and engineer them through several rounds of genetic modification. We propose to enhance the activity of CAR-NK cells by co-expressing the secreted blockers of CD47/SIRPa interaction (CD47-specific mAb, scFv, and CD47 ectodomain). These molecules are known to stimulate the phagocytosis of cancer cells, and the supernatants from CAR-YT cells secreting the above CD47/SIRPa blockers (ECARs) enhanced the phagocytic activity of primary human macrophages against Jurkat cells. This approach opens an opportunity to use ECARs in an allogeneic setting and to target bystander cancer cells that are otherwise missed by the CAR. This study was supported by the grant from the Russian Science Foundation # 16-14-10237.

Cancer - Oncolytic Viruses I

427. Tumor Specific T Cells Generated by Cytokine-Armed Oncolytic Vaccinia Virus Represent a New Therapeutic Strategy for Adoptive T Cell Transfer

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Background: Immunotherapy is rapidly evolving and fighting cancer by re-activating the patient immune system presents an attractive therapeutic strategy for gastrointestinal cancer besides standard treatments as surgery, chemotherapy and radiotherapy. Immunotherapeutic strategies for gastrointestinal malignancies have to overcome poor lymphocyte infiltration and a highly immunosuppressive tumor microenvironment. The application of oncolytic vaccinia viruses with induction of immunogenic cell death offers an effective strategy to overcome less immunogenic tumors and to induce an efficient anti-tumor T cell response. Viral mediated cell death results in the release of potent danger signals and cross presentation of tumor-associated antigens, resulting in potent antitumor innate and adaptive immunity. We present for the first time a new concept to promote intratumoral T cell infiltration for generation of tumor-specific T cells for cancer therapy via adoptive T cell transfer. **Methods:** MC38 s.c. tumor bearing mice have been treated with IL-2-armed vaccinia virus (vvDD-IL2), control virus (vvDD) or PBS. Lymphocyte infiltration of viral or PBS treated tumors has been quantified by immunofluorescence. Additionally, MC38 tumor infiltrating T cells have been tested for tumor specificity using a co-culture assay with MC38 cells versus irrelevant target cells as B16 cancer cells or naïve splenocytes. MC38 tumor-specific response has been measured by IFN-γ release (IFN-γ -ELISPOT) and 4-1BB expression (flow cytometry). For adoptive T cell transfer, T cells derived from vvDD-IL2-treated MC38 tumor bearing mice versus naïve T cells as control have been expanded ex vivo and transferred into MC38-luc i.p. tumor bearing mice. All animals received 5 Gy irradiation for lymphodepletion and IL-2 support. Tumor progression has been monitored using live animal bioluminescence imaging. **Results:** Intratumoral application of vvDD-IL2 promoted a significant $CD8^+$ T cell infiltration into the tumor when compared to PBS treatment ($p < 0.001$). The IL-2 armed vaccinia virus induced a significant increase of tumor reactive $CD8^+$ T cells compared to control virus ($p < 0.05$), IL-2 ($p < 0.001$) or PBS treatment ($p < 0.0001$). The vvDD-IL2 induced T cells presented a highly tumor specific IFN-γ response and 4-1BB expression and retained their therapeutic potential when expanded *ex vivo* and transferred into tumor bearing mice. Adoptively transfer of the vvDD-IL2 induced tumor-specific T cells leads to significant tumor regression and long-term survival in MC38 tumor bearing mice. **Conclusion:** Our report presents a new therapeutic concept to promote intratumoral T cell infiltration and to generate tumor-specific T cells for adoptive T cell transfer. We aim to translate our strategy from bench to bedside in the near future.

428. Toca 511-Mediated Prodrug Activator Gene Therapy: A Promising Therapeutic Strategy for Ovarian Cancer

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Ovarian cancer causes more deaths in the U.S. than any other malignancy of the female reproductive system, and new treatment approaches are needed. *Vocimagene amiretrorepvec* ('Toca 511'), a tumor-selective retroviral replicating vector (RRV) encoding yeast cytosine deaminase (CD) which converts the prodrug 5-fluorocytosine (5-FC) into the chemotherapy drug 5-fluorouracil (5-FU), is currently being evaluated in an international Phase III trial (NCT02414165) for recurrent glioma, and a Phase I trial (NCT02576665) evaluating systemic delivery of Toca 511 to several tumor types is now underway, so it is timely to consider applying this approach to gynecologic malignancies. Here we report on the first preclinical studies to evaluate RRV-mediated prodrug activator gene therapy in experimental models of ovarian cancer. First, *in vitro* replication of RRV expressing the green fluorescent protein reporter gene (RRV-GFP) was monitored by flow cytometry. Efficient RRV replication and spread was observed *in vitro* in both established and primary ovarian cancer cell lines with >80~90% transduction achieved by Day 9-12. Next, cytotoxicity was quantitated by MTS assay after 5-FC treatment of RRV-GFP vs. RRV-CD (Toca 511)-transduced ovarian cancer cells. After RRV-CD transduction, significant reduction of cell viability was observed in a 5-FC prodrug dose-dependent manner. *In vivo* RRV-GFP replication kinetics were also examined by flow cytometry following intraperitoneal (IP) vector injection in SKOV3-IP peritoneal carcinomatosis models. Tumor-selective vector replication and spread were confirmed *in vivo*, with tumor transduction levels increasing over time in a vector dose-dependent manner, reaching >90% within 3 weeks post-vector inoculation with a single high-dose bolus IP vector injection in SKOV3-IP peritoneal carcinomatosis models. Finally, therapeutic efficacy of RRV-CD (Toca 511)-mediated prodrug activator gene therapy was examined following 5-FC prodrug administration by bioluminescence imaging and Kaplan-Meier analysis. Reduced tumor burden and significantly increased survival was observed associated with higher tumor transduction levels after 5-FC treatment in the RRV-CD (Toca 511)-transduced group, as compared to untreated controls and control groups treated with vector only or prodrug only. In conclusion, these results using Toca 511/5-FC prodrug activator gene therapy in preclinical models of disseminated ovarian cancer support future efforts toward clinical translation.

429. The Combinatory Treatment of the Oncolytic Adenovirus ONCOS-102 with Anti PD-1 (Keytruda®) Show Synergistic Anti-Tumor Effect in Humanized A2058 Melanoma huNOG Mouse Model

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INTRODUCTION With clinical introductions of checkpoint inhibitors (CPIs), both response rates (RR) and overall survival (OS) have been improved in advanced melanoma. However, despite significant clinical advancements, at least 40% of patients do not respond to CPIs. Adenoviruses are excellent immunotherapeutic agents with a unique ability to both prime and boost immune responses. ONCOS-102 is a serotype 5, human, double-targeted oncolytic adenovirus with a chimeric 5/3 capsid for enhanced cancer cell transduction. It has a 24 bp deletion in the Rb binding site of the E1A gene for cancer-cell restricted replication. The virus codes for human granulocyte macrophage colony-stimulating factor (GM-CSF) to enhance anti-tumor immunity. ONCOS-102 induced both innate and adaptive immune activation correlated with overall survival (OS) in a phase I study of different types of treatment refractory solid tumors. In the same study PD-L1 was upregulated in tumor lesions. There may be further enhanced clinical benefit by combining with CPIs as inhibition of immune checkpoints is crucial for efficient immunotherapy and immune responses stimulated by oncolytic viruses exhibit antitumor effects. Therefore, we have performed a series of pre-clinical studies to investigate any potential enhanced anticancer properties when combining ONCOS-102 and Keytruda®. **MATERIAL AND METHODS** To study the efficacy of combinatory therapy of ONCOS-102 and anti-PD-1 in melanoma, we have developed a humanized A2058 melanoma huNOG mouse model. The NOG mouse strain was engrafted with cord blood-derived CD34+ hematopoietic stem cells after chemical myeloablative treatment. Fourteen weeks after cell injection, engraftment level was monitored with the analysis of human CD45+ cells among total blood leukocytes. Humanization rate was defined as the ratio of circulating hCD45+/total CD45+ (mCD45+hCD45). 60 humanized NOG mice were engrafted with A2058 tumor cells and randomized in 8 groups. Different treatment regiments of the ONCOS-102, Keytruda® and their combinations were investigated. Throughout the study tumor volume and body weight was monitored and sacrifice was scheduled on day 40. Immune cell infiltration and PD-L1 expression in a tumor was analyzed by flow cytometry. **RESULTS** ONCOS-102 significantly reduced tumor volume by 52% while the treatment with Keytruda® did not show therapeutic effect compared to vehicle. The combinatory therapy with the virus and Keytruda® showed a reduction of 69% compared to vehicle ($p=0.004$). The treatment with ONCOS-102 increased hCD3+ and hCD8+ T cells infiltration where as Keytruda® alone did not have an effect on T cell recruitment within the tumor. The highest increase of CD8+ infiltrating T cells was observed in the combinatory group of ONCOS-102 and Keytruda® ($p<0.05$). **CONCLUSIONS** This study demonstrate synergism between ONCOS-102 and Keytruda®

and support the scientific rationale for the ongoing clinical study of ONCOS-102 and Keytruda® in CPI refractory advanced melanoma (NCT03003676).

430. Neural Stem Cell Mediated Delivery of AR2011 Oncolytic Virus for Ovarian Cancer

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Ovarian cancer is the most lethal gynecologic malignancy, afflicting approximately 22,000 women per year in the U.S. Once it has metastasized to the abdominal cavity (stage III), patients have only a 34% 5-year survival rate following standard treatment with surgical debulking and combination chemotherapy. Oncolytic virotherapy is a promising novel approach that can induce cancer cell death irrespective of radio- or chemoresistance, and also stimulate immune system recognition of cancer cells by exposing tumor antigens upon lysis. Although clinical trials to date have demonstrated safety, the efficacy of this approach has been limited by delivery hurdles including rapid inactivation by the immune system, poor viral penetration of tumors, and an inability of the virus to effectively reach invasive metastatic foci separated by normal tissue. Inherently tumor-tropic neural stem cells (NSCs) have the ability to penetration metastases, making them an ideal cell carrier to overcome these hurdles. In particular, the clonal human NSC line used here (HB1.F3.CD21) enables reproducible viral loading, non-immunogenicity, and chromosomal stability, with demonstrated clinical safety in first-in-human brain tumor trials. We previously reported NSC distribution to peritoneal ovarian metastases in an immunodeficient model. We now demonstrate NSC distribution to intraperitoneal (IP) ID8.Renilla.eGFP ovarian cancer metastases in a C59Bl/6 immunocompetent mouse model. This allows us to assess their ability to provide protection from immune-mediated viral clearance and neutralization, while selectively delivering oncolytic viruses to ovarian tumor foci. The oncolytic adenovirus used in these studies, AR2011, replicates under the control of the Secreted Protein Acidic Rich in Cysteine (SPARC) promoter. SPARC is overexpressed not only in tumor cells, but also in tumor-associated stroma, enabling efficient viral spread throughout the tumor and its microenvironment. AR2011 also contains enhancer elements that respond to tumor conditions of hypoxia and inflammation. Significant tumor killing of ID8 ovarian cancer cells was observed *in vitro* after 5 days of co-culture with NSC.AR2011 (at a ratio of 1000:1). NSCs also protected the oncolytic activity of AR2011 when cultured in the presence of ovarian cancer patient ascites fluid, which was confirmed to have neutralizing antibodies against adenovirus. For *in vivo* NSC biodistribution studies, NSC.qtracker 605, or NSC.qtracker 655 were injected IP. Two days later, 3D block-face cryo-images of harvested mice were created to visualize NSC biodistribution. For oncoviral efficacy studies, 5E6 of either NSC.AR2011 or free AR2011 were administered IP weekly for 3 weeks, with cisplatin and no treatment controls, and followed for long-term survival, monitoring tumor progression with serial BLI. *In vivo* results demonstrate IP NSC.AR2011 seeding of virus at the majority of established ovarian tumor metastases. Viral distribution was confirmed via IHC and qPCR. Comparative *in vivo* efficacy studies are in progress, with and without a PD-L1 checkpoint inhibitor to potentially enhance the oncolytic virotherapy with an additional immune component. We expect NSC-mediated AR2011 treatment +/- PD-L1 antibody to improve long-term survival as compared to standard of care chemotherapy regimens, without the associated off-target toxicities. We aim to demonstrate preclinical efficacy and safety, to streamline this NSC.AR2011 approach to clinical trials for in patients suffering from stage III ovarian cancer.

431. Transient Interferon Suppression Renders Nerve Sheath Sarcomas Susceptible to Oncolytic HSV Immunotherapy

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Malignant Peripheral Nerve Sheath tumors (MPNSTs) are aggressive soft tissue sarcomas resistant to most cancer treatments. Surgical resection remains the primary treatment; however, this is often incomplete and leads to high mortality and morbidity rates. Encouraging preclinical and clinical trial results has spurred interest in a virotherapeutic approach for these sarcomas. Both direct lysis of tumor cells and immune mediated anti-tumor activity contribute to oncolytic Herpes simplex viruses (oHSV) therapy. We previously showed that basal interferon (IFN) signaling increases interferon stimulated gene (ISG) expression, restricting viral replication in almost 50% of MPNSTs (Jackson 2016). The FDA approved drug Ruxolitinib (RUX) temporarily suppresses constitutively active STAT-signaling and renders the tumor cells susceptible to oHSV infection in cell culture. In these studies, we translated our *in vitro* results into a syngeneic MPNST tumor model. Consistent with our previous results, murine MPNSTs exhibit a similar IFN- and ISG-mediated oHSV resistance and virotherapy alone provides no antitumor benefit *in vivo*. However, when mice are pre-treated with Ruxolitinib, this reduces ISG accumulation and improves viral replication. Resetting basal IFN signaling in the tumor also changes the oHSV-induced immune-mediated response: enhancing CD8 Cytotoxic T cell (CTL) activation after oHSV treatment. Our studies also show that this CD8 population is indispensable for RUX+oHSV antitumor benefit. In summary, these studies show that transient JAK inhibition can expand the number of tumors amenable to oHSV therapy and not only improves oHSV replication in the tumor but also enhances OV-induced immune mediated anti-tumor effect.

432. Anti-Tumor and Anti-Metastatic Effect of CD133-Targeted Oncolytic Adenovirus in Colorectal Cancer

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Colorectal cancer (CRC) is the third most common cancer in the world, and about 50% of patients relapse after treatment. Cancer stem cells (CSCs) have contribution to recurrence, metastasis and

chemotherapy resistant of CRC. CD133 (Prominin-1), a member of the transmembrane glycoprotein family, is a marker of CSCs in several cancers including CRC: its expression correlates with recurrence, metastases and chemotherapy resistance, as well as poor prognosis in CRC. It is therefore reasonable to develop a CSC-directed CRC therapeutic strategy by employing CD133 as a target molecule. Recently, we have established a method for isolating transductionally-targeted infectivity-selective adenovirus by high-throughput screening. Using this adenovirus library screening system, we isolated the CD133-specific Oncolytic Adenovirus (OAd) and tested the oncolytic activity of CD133-targeted OAd (CD133-OAd) in both *in vitro* and *in vivo,* with and without irradiation which induces CD133 expression. The infectivity-selective OAd (ISOAd) with CD133-targeting motif (TYML motif) selectively infected $CD133^+$ CRC cell lines and lysed them efficiently. In the context of modulation of stemness, CD133-OAd inhibited colony formation in vitro. In tumor formation assay in nude mice, treatment with CD133-OAd prior to tumor inoculation inhibited the establishment of tumor of $CD133^+$ CRC cell lines. Intra-tumor (i.t.) administration of CD133-OAd into established subcutaneous tumor exhibited significantly stronger antitumor effect compared to OAd without targeting. We have reported that the irradiation increases CD133 expression in CRC cells, and the replication of CD133-OAd increased significantly after irradiation. In athymic nude mice, treatment of irradiated cells with CD133-OAd abolished tumor-forming capacity, compared to cells without irradiated and cells treated with radiation alone (5% vs 94% and 5% vs 73% respectively, $p<0.0001$). When the antitumor effect of CD133-OAd was analyzed on subcutaneously established tumors, treatment with radiation and CD133-OAd significantly reduced tumor growth compared to no treatment and treatment with radiation only (both $p<0.0001$). In addition to the effect on the main tumor, CD133-OAd suppressed liver metastasis of CRC. The mice injected with human CRC cells pretreated with CD133-OAd combined with irradiation had significantly lower incidence of liver metastasis compared with the untreated control group or the groups received irradiation or CD133-OAd treatment alone. Our CD133-targeted ISOAd is effective for cytotoxic killing, reduces tumor formation, and mitigates tumor growth in radiation resistant CRC cells. This targeted OAd therapy may be applicable to address therapeutic resistance and prevent the establishment of recurrent colorectal cancer.

433. A Third Generation Oncolytic HSV-1 G47Δ Enhances the Efficacy of Radiofrequency Ablation Therapy of Hepatocellular Carcinoma

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Introduction:G47Δ is a triple-mutated, third generation oncolytic herpes simplex virus type 1 (HSV-1) that can selectively replicate in and kill tumor cells. Radiofrequency ablation (RFA) is a standard local treatment for hepatocellular carcinoma (HCC). Although recurrence at the site of RFA is infrequent, multiple remote recurrences within the liver often occur. Recently, it is reported that tumor cell destruction by certain types of therapy, including oncolytic HSV-1, can elicit systemic antitumor immunity. Here, we hypothesized that treatment of HCC by G47Δ may enhance the therapeutic efficacy of RFA through augmentation of antitumor immunity, and may prevent recurrences. Methods: Subcutaneous tumors were generated by implanting poorly immunogenic Neuro2a cells to the flank of syngeneic A/J mice. The tumors were then treated with intratumoral injections with G47Δ (2×10^6 pfu) or mock on days 0, 2 and 4, followed by RFA on day 6. RFA was performed using StarMed VIVA RF system at a power of 5 watt for 60 to 80 seconds, using 5 millimeters long needle directly inserted to the tumor. The treated primary tumors were totally eliminated with the combination of G47Δ and RFA and no local recurrence was observed by day 19. To mimic a remote recurrence, Neuro2a cells were then implanted to the contralateral flank of the treated mice on the same day of RFA, and the tumor development was observed. Tumors larger than $50mm^3$ were defined as engrafted and the rate of engraftment was compared. In a separate experiment without rechallenge, antitumor immunity was evaluated using ELISpot assay on day 25 by counting Neuro2a reactive, interferon gamma (IFN-γ) secreting splenocytes. To assess the population of tumor infiltrating lymphocytes (TIL) and splenocytes, flow cytometric analysis was performed and the numbers of CD8(+) T cell, CD4(+) T cell, NK cell, regulatory T cell (Treg), Helper T cell and myeloid-derived suppressor cell (MDSC) in CD45(+) lymphocyte, as well as CD8/Treg ratio were analyzed. Results: All mice in the mock+RFA group formed tumors from rechallenged Neuro2a cells, while four out of seven mice in the G47Δ+RFA group did: The engraftment rate was significantly different ($p = 0.03$). ELISpot assay showed that the mean number of IFN-γ spots was significantly increased ($p < 0.0001$) in the G47Δ+RFA group (95 spots/well) compared with the mock+RFA group (1.8 spots/well). The proportion of CD8+/CD45+ T cell, and CD8+/Treg T cell in the contralateral tumors and the spleen were significantly higher in the G47Δ+RFA group (TIL; $p = 0.018$ and $p = 0.047$, spleen; $p = 0.026$ and $p = 0.016$). The proportion of MDSC/CD45 T cell in the contralateral tumors was significantly lower in the G47Δ+RFA group ($p = 0.030$). With or without G47Δ, no significant difference was observed for CD4/CD45, NK/CD45 and Helper T/CD45 ratio. Conclusion: These results indicate that intratumoral G47Δ administration prior to RFA may promote the induction of systemic antitumor immunity. The combination of G47Δ and RFA could be an effective regimen for the treatment of HCC.

434. IFITM1 Plays an Important Role in Determining Resistance of Normal MSCs to Oncolytic Measles Virus

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Vaccine strain measles virus (MV) is oncolytic in numerous models of malignancy. The mechanism behind the selectivity of MV for transformed cells is poorly understood. To investigate further, an established step-wise model of cellular transformation was used; in which progressive oncogenic hits were stably and additively expressed in human bone marrow derived mesenchymal stromal cells (Funes *et al.,* 2007). The most highly transformed cells (5H) were more permissive to

oncolytic MV infection than any of the less transformed counterparts, with significantly greater viral titres. MV-induced cell-death increased progressively with progressive transformation. This was not explained by differences in MV receptors CD46, SLAM or nectin-4 expression. Investigation of anti-viral type 1 IFN response in this model 24 and 48 hours post MV infection (hpi) by ELISA demonstrated a robust induction of IFNβ (to a lesser extent IFNα) in hTERT cells, which was significantly and progressively reduced in 3H, 4+V and 5H according to level of transformation, suggesting that defective IFN pathway is a potential mechanism for the enhanced MV permissiveness observed in transformed cells. Moreover, examination of the integrity of the RLR signalling pathway, which triggers IFNα/β production, revealed that expression levels of RIG-I and MDA-5, determined by RQ-PCR at 12, 24 and 48 hpi, were lowest in 5H and highest in hTERT cells, proposing a role for the RLR pathway in MV-mediated oncolysis. To confirm the biological relevance of IFN production in MV-permissiveness, 5H cells were exogenously treated with IFNα/β. However, this did not render the cells resistant to MV infection and compensate for the lack of native IFN production. In order to identify genes associated with resistance to MV infection, whole genome expression profiling was performed using the Illumina NextSeq 500 platform. Gene expression profiles (GEP) of hTERT and 5H cells showed that interferon-stimulated genes (ISGs) were significantly down-regulated with cellular transformation, suggesting that hTERT cells are in a pre-existing antiviral state and thus more capable of fighting MV infection compared to their malignant counterparts. Moreover, GEP showed up-regulation of a subset of ISGs in response to MV infection, with hTERT cells showing larger fold increases compared to minimal up-regulation in 5H cells. Amongst the ISGs identified, IFITM1 demonstrated the most prominent change with a 183-fold reduction in 5H cells compared to hTERT cells at basal levels. IFITM1 belongs to the IFITM (interferon-induced-transmembrane) family proteins of which IFITM1, IFITM2 and IFITM3 have been implicated as viral restriction factors, but not for MV. MV infection strongly induced IFITM1 expression in hTERTs (3.6-fold) compared to a weaker induction in 5H cells (1.2-fold). Induction of IFITM1 protein expression was confirmed by immunoblotting at 24hpi. To confirm the role of IFITM1 on susceptibility to MV infection, we are currently assessing the effects of overexpression in the susceptible cell line, 5H. Altogether, our data suggests that basal innate immune responses are critical biomarkers for oncolytic measles virotherapy. IFITM1, in particular, seems to play a crucial role in MV permissiveness and may be a newly-identified restriction factor for MV.

435. Oncolytic Adenoviruses Encapsulated into the Extracellular Vesicles as Carriers for Cancer Drug Delivery

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Introduction: Cancer standard of care is commonly a combination of surgery with chemotherapy and/or radiotherapy. However, in advanced cancer patients this approach is inefficient and may cause many side effects, including severe complications and even death. Oncolytic viruses exhibit different anti-cancer mechanism compared to conventional therapies, since they are specifically engineered to preferentially infect, replicate in and kill cancer cells instead of normal cells where their normal functions are restricted. Additionally oncolytic viruses are often administered intratumorally, thus many solid tumors cannot be treated using this approach. Extracellular vesicles (EVs), which are naturally occurring cargo delivery agents have a potential to be used as vehicles for drug delivery. Therefore we hypothesized that oncolytic adenoviruses encapsulated into EVs loaded with chemotherapeutic drugs should enhance specific drug delivery for tumor targeting, and thus improve efficacy of cancer treatment. Here, we investigated the systemic delivery of oncolytic adenoviruses and paclitaxel encapsulated in extracellular vesicles formulation in order to utilize them as carriers for cancer drug delivery. **Methods:** The *in vivo* efficacy of EV-Virus-Paclitaxel complex was tested in Balb/c nude mice after intravenous injection. Transcriptomic analysis carried out on the explanted xenografts from the different treatment groups was used to examine synergistic effect observed between the EV delivered virus and Paclitaxel into the tumors. **Results:** We found that the obtained EV-Virus and EV-Virus-Paclitaxel formulations reduced the *in vivo* tumor growth in xenograft model of human lung cancer. Indeed, combined treatment of oncolytic adenovirus and paclitaxel encapsulated in EV showed synergistic anticancer effects both *in vitro* and *in vivo* lung cancer models. Transcriptomic analysis carried out on the explanted xenografts from the different treatment groups failed to show a simple additive effect of paclitaxel on the genetic programs triggered by the EV virus administration indicating that a *de novo* genetic program is triggered by the presence of the encapsulated paclitaxel. **Conclusions**: Our work provides a promising approach combining anticancer drugs and viral therapies by intravenous EV delivery as new therapeutic strategy aimed at treating lung cancer.

436. The Porcine Model for Onocolytic Adenovirus-Based Therapy

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Oncolytic adenoviruses (Ad) are promising tools in the development of cancer therapeutics. A majority of Ad-based therapies utilize serotypes of species C, with Adenovirus type 5 (Ad5) being the most commonly employed. Previously, clinical trials have demonstrated the low efficiency of Ad5 vectors, mainly due to absence of the Ad5

primary receptor (Coxsackie Adenovirus Receptor, CAR) in cancer cells. Engineering Ad vectors utilizing the species B (Ad3, Ad35, Ad11) receptors have greatly improved the oncolytic potential of Ad-based therapies. However, the lack of a viable animal model has impeded clinical translation of these tropism-modified vectors. Mouse models are insufficient because Ad does not replicate in murine tissue. Non-human apes are not feasible due to availability and cost. Cotton rats and Syrian hamsters, although permissive of Ad5 replication, are not suitable for Ad3-, Ad35-, and Ad11-retargeted vectors due to the lack of species B primary receptors (CD46 and desmoglein 2) in rodent systems. In this study, we explored pigs as a model to study performance of the group B oncolytic adenoviruses by employing the fiber-modified Ad5/Ad3 chimeric vector. As a control, the Ad5 fiber-unmodified virus was used. We analyzed binding, gene transfer, replication, and cytolytic ability of Ad5 and Ad5/Ad3 in various non-human cell lines (porcine, hamster, murine, rat, canine) and isolated swine ductal cells. Our data revealed that among all tested cell lines, only porcine established (PK15 and PTK75) and primary cells were supportive of binding Ad5/Ad3. Murine and hamster cells supported binding of the Ad5 vector; however, they failed to facilitate Ad5/Ad3 binding. Furthermore, analysis of viral binding in swine cells and CAR-negative hamster CHO cells with the addition of pig blood factor X significantly increased binding of Ad5, a phenomenon that was also observed with human blood factors XI and X. Adenoviral binding to pig factor X did not significantly increase binding ability of Ad5/Ad3. Cell viability analyses with Ad5/Ad3 revealed cell death only in swine cells and no death in the rodent and canine models, further corroborating porcine cells as the only non-human model to permit Ad5/Ad3 vector replication. Interestingly, Ad5/Ad3 outperformed Ad5 in its cytolytic effect in porcine established and primary cells. These results positively correlated with Ad5/Ad3 replication-dependent gene transfer. In summary, the *in vitro* studies revealed only porcine cells to be supportive of both binding and replication of Ad5/Ad3. The adenoviral vectors were subsequently evaluated *in vivo*. Immunocompetent Yorkshire pigs were systemically injected with a single dose of Ad5 and Ad5/Ad3. Primary organs were collected 1 and 7 days post-infection. Quantitative PCR analyses and immunohistochemistry of viral DNA revealed Ad5 and Ad5/Ad3 DNA in the lungs, spleen, and lymph nodes. Replication-dependent Luc expression was also observed in these tissue samples suggesting active viral replication. The quantity of viral DNA in other tissue such as the liver, kidneys, and pancreas was negligible. Of note, no severe Ad-related adverse effects were observed in the pigs. The results of our *in vitro* and *in vivo* studies indicate that pigs are a valuable model to assess safety and bio-distribution of conventional Ad5 and Ad3-retargeted adenoviral vectors.

437. Cytotoxicity of Replication-Competent Adenoviruses Defective of E1B55kDa Molecules is Irrelevant to P53 Expression, but Increases with a P53-Augmenting MDM2 Inhibitor through DNA Damages and Enhanced Viral Propagations in Mesothelioma with the Wild-Type P53 Genotype

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Recent whole exome sequencing data showed that a majority of clinical specimens from mesothelioma patients was defective of the INK4A/ARF region due to the genomic deletion or methylation of the regulatory region. The defect consequently induced loss of p53 functions since MDM2 encoded in the region was critical for p53 ubiquitination and the degradation process. Reactivation of endogenous p53 is therefore important for mesothelioma treatment because the p53 genotype was wild-type in most of the cases. We showed that oncolytic adenoviruses lacking of E1B55kDa (Ad-E1B) molecules increased endogenous p53 levels and induced cell death in mesothelioma through apoptosis. Knock-down of p53 with si-RNA however did not influence the cytotoxicity, indicating that the Ad-E1B-induced cell death was irrelevant to p53 expression but mediated by the viral replications. On the other hand, nutlin-3a, a MDM2 inhibitor, augmented endogenous p53 levels and cell cycle arrest at G1 phase in mesothelioma cells with the wild-type p53 but not in those with mutated p53 genotype. We investigated a possible role of up-regulated endogenous p53 in the Ad-E1B-mediated cytotoxicity in mesothelioma. Combination of nutlin-3a and the oncolytic Ad augmented ATM/Chk2 but not ATR/Chk1 phosphorylation, and increased NF-1 expression in mesothelioma cells with the wild-type but not in those with mutated p53. The combinatory use enhanced production of the viral progenies and achieved synergistic cytotoxicity only in wild-type p53 mesothelioma. These data collectively indicated that the up-regulation but not down-regulation of p53 play a critical role in virotherapy in the majority of mesothelioma and that enhanced p53 was favorable to Ad replications-induced cytotoxicity through increased DNA damages and production of viral progenies.

438. Impact of the Interferon Response on Intratumoral Adaptation of Oncolytic Picornaviruses

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Oncolytic virotherapy is a therapeutic approach that harnesses the cytotoxic potential of native or genetically modified viruses to selectively target tumor cells and prime antitumor immunity. The presence of a functional interferon-mediated antiviral response in the tumor microenvironment capable of counteracting early stages of viral infection can be a limitation to successful oncolytic virotherapy. Picornaviruses are small, non-enveloped, single-stranded, positive-sense RNA viruses that are gaining momentum as oncolytic agents thanks to their broad species and tissue tropism, simple genome structure, high yield, rapid replication and suitability for microRNA targeting. Due to the low replication fidelity of their viral polymerase and the absence of proofreading and editing mechanisms, picornaviruses exist as quasispecies, a dynamic and organized distribution of non-identical but related genomes dominated by a master sequence. The intrinsic diversity of the quasispecies is a key factor for adaptation to the multiple selective pressures imposed by the rapidly changing environment in which viruses replicate. More specifically, the ability to overcome unique antiviral microenvironments dictated by the type I interferon response determines pathogenesis, viral tropism, replication selectivity and ultimately drives tissue-specific adaptation of picornaviruses. Two picornaviruses - a live attenuated strain of Mengovirus carrying a deletion in the polycytidine trait (MC_{24}) and wild type coxsackievirus A21 (CVA21) - have been validated as potent and effective oncolytic agents in our lab and their safety profiles improved through microRNA detargeting. We now seek to investigate the role played by the type I IFN antiviral response of the tumor microenvironment in shaping MC_{24} and CVA21 intratumoral adaptation. We aim to describe the impact that tumor-specific IFN signatures have on mutational patterns across the genome and on fitness landscapes. We will also identify mutations that enhance interferon-resistance and characterize their effects on viral replication and the oncolytic potential of MC_{24} and CVA21. This analysis will highlight the functional and structural constraints to picornavirus genome modifications, correlate IFN-resistance with specific mutations in the context of different IFN evasion systems, address the problem of the presence of an intact tumor antiviral system on the success of oncolytic virotherapy and provide guidance for the rational design of better and safer oncolytic tools.

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439. Tumor Mediated CAR Inhibition at the Immune Synapse Takes Place by Disruption of Polarization of the Microtubule Organizing Center

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Problem: Direct inhibition of immune cells by the tumor or tumor microenvironment is still a major limitation of immunotherapy. We reasoned that interdiction of an effective immune synapse (IS) between effector T cell and tumor target cell is a key component of that failure. Here we investigate the effect of tumor mediated inhibition of CAR T cell cytotoxic function in the context of CD19 and HER2 CARs for liquid and solid tumors respectively. We discover a common inhibition mechanism that affects CAR molecules signaling through a CD28 costimulatory endodomain. **Background**: Cytotoxic T cells (CTL) use a tightly controlled & hierarchical process using specific antigen receptors to engage, organize, polarize and secrete specialized lytic machinery onto a diseased cell to kill it. We have previously demonstrated that like TCRs, CARs engaging a tumor cell mobilize a specialized lysosome related organelle called the lytic granule that contains the pore forming molecule perforin and proteolytic enzymes to be secreted onto the target cells (Mukherjee et al., 2017). Upon recognition of a tumor cell by a CAR T cell the lytic granules actively converge around the microtubule organizing center (MTOC) after which, they are polarized along with the MTOC towards the tumor cell to a special interface known as the immune synapse (IS) & secreted onto the target cell in a process called "degranulation". Failure of any of these steps have been shown to be disruptive to effective cytotoxicity and hence to CAR T cell functionality (Hegde, Mukherjee et al., JCI, 2016). **Approach**: Here we used a high-resolution subcellular quantitative imaging toolbox including confocal and stimulated emission depletion (STED) microscopy to evaluate the structural and functional components of the CAR immune synapse using CD19 and HER 2 specific CARs and an array of liquid and solid tumor targets for each CAR type. With these approaches, we were able to achieve unprecedented levels of subcellular resolution of the CAR IS and evaluate the co-ordination of pro and inhibitory signals thereof. We investigated differences in synapse formation by these CARs with susceptible targets and targets with higher resistance to cytotoxicity. We used F-actin polymerization at the IS and polarization of the MTOC to the IS as two key cytotoxic parameters for evaluating effective cytotoxic function by the CARs. **Results**: We found that while both 4-1BB.Z and CD28.Z CARs express high levels of surface PD-1, but only in CD28.Z CARs PD-1 is recruited to the CAR immune synapse, whereas in 4-1BB.Z CARs, PD-1 remains uniformly distributed on the CAR surface also in CARs conjugated with tumor cells. As a result, 28.Z CAR T cells are especially susceptible to inhibition by tumors expressing surface PD-L1. Engagement of PD-1

and PD-L1 at the CAR IS results in reduced accumulation of PKC theta, a key CAR downstream signaling component, at the CAR IS. This in turn, leads to inefficient polarization of the MTOC to the CAR IS and delayed or ineffective CAR mediated killing. **Conclusion**: Collectively, these results indicate that CD28.Z CAR T cells are more likely to be down-regulated by inhibitory tumor or tumor microenvironments that enhance the PD-1/PD-L1 axis, suggesting that functional blockade of the PD-1/PD-L1 interaction may preferentially enhance the anti-tumor activity of CD28.z CAR T cells.

440. Reprogramming CD4 T Cells into Cytotoxic CD8 Cells by Forced Expression of CD8αβ and Class I Restricted T Cell Receptors

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Introduction: T cell receptors (TCRs) targeting MHC class I restricted tumor-associated antigens (TAAs) are characterized by low avidity and depend on the CD8 co-receptor for T-cell activation. The interaction of CD4 and CD8 T cells is necessary for optimal immune responses. We hypothesized that forced expression of CD8αβ with an HLA Class I restricted TCR would enable CD4 T cells to recognize and kill target cells expressing the cognate Class I restricted antigen while preserving their beneficial CD4 helper properties. We now describe improved in vivo anti-tumor function after adoptive transfer of tumor-specific CD4 and CD8 T cells equipped with a single TCR. **Methods:** CD4 and CD8 T cells were separated from healthy donors and retrovirally transduced with a panel of 4 TCRs±CD8αβ targeting survivin or PRAME. Cells were analyzed by flow cytometry, IFNγ ELISPOTs, cytotoxicity assays, cytokine multiplex, live cell time-lapse microscopy and in a xenograft mouse model. **Results:** CD4 and CD8 T cells were efficiently transduced. CD8α levels in TCR+CD8αβ+ (T8) CD4 cells were similar to native TCR+ CD8 cells (CD8α MFI: 4956±2014 vs 4631±1714, n=7, mean±SD, p=NS). CD8β levels in T8+ CD4 cells were slightly lower than native TCR+ CD8 cells, (CD8β MFI: 980±560 vs 1845±653, n=7, p=0.004), but higher in T8+ CD8 cells (6189±2774, n=7, p=0.007). TCR+ CD4 T cells only recognized the targeted survivin epitope when CD8αβ was co-expressed (Dextramer MFI, TCR vs T8 CD4: 111±85 vs 2267±2479, CD8: 3425±552 vs 4164±2627). The avidity of T8+ CD4 T cells was comparable to TCR+ native CD8 T cells in 2 of the 4 TCRs tested based on IFNγ ELISPOT. When using a survivin-specific TCR, the IFNγ response to the HLA-A*02:01+survivin+ target cell line BV173 was comparable between T8+ CD4 and TCR+ CD8 T cells (TCR vs T8 CD4 41±42 vs 487±178, n=7, p=0.001; CD8 200±171 vs 327±123 SFU/10^5 cells, n=7, p=NS) and they efficiently killed BV173 cells in vitro in serial co-culture assays. T8+ CD4 T cells produced cytotoxic Th1 cytokines including IFNγ, TNFα, perforin, granzyme A and B and also the Th2 cytokine IL10, compared to native T8+ CD8 T cells that exclusively produced Th1 cytokines. To assess whether reprogramming of CD4 T cells to cytotoxic effector cells was due to the transgenic co-expression of CD8αβ, we analyzed LCK phosphorylation after tumor stimulation. We found increased pLCK levels (TCR vs T8; CD4 p=0.009, CD8 p=0.03, n=4), indicating that the CD8 co-receptor not only provides stability to the TCR-pMHC complex but also enhances early TCR signaling events. The analysis of single cell killing kinetics by live time-lapse microscopy confirmed that T8+ CD4 T cells become cytotoxic with similar killing behavior as TCR+ native CD8 T cells. Finally, we tested the in vivo anti-tumor function. BV173 leukemia was significantly controlled in NSG mice treated with T8+ CD4 T cells on day 35 (TCR vs T8 p=0.001, n=5). In mice treated with CD8 T cells, TCR alone provided significant leukemia control (control vs TCR, p=0.0002). CD8αβ co-transfer further enhanced this effect (TCR vs T8, p=0.01, n=5). **Conclusions:** Forced expression of CD8αβ in class I TCR-transgenic CD4 T cells produces a cytotoxic CD8 T cell phenotype, leading to enhanced anti-tumor function. Native CD8 T cell function was also enhanced in vivo. This approach provides us with the means of redirecting both CD4 and CD8 T cells to a TAA using a single cytotoxic receptor and thereby improve their in vivo anti-tumor function.

441. Gene-Modified Hematopoietic Stem and Progenitor Cells Engraft Systemically, and Give Rise to CNS-Associated Microglia

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Introduction HIV reservoirs are a primary barrier to HIV cure, leading to persistence of latently infected cells in multiple, physiologically distinct compartments. Reservoirs in the central nervous system (CNS) pose a particular challenge, due to inefficient delivery of many therapies to this site. Hematopoietic Stem and Progenitor Cell (HSPC) gene therapy holds great promise in a number of hematopoietic-origin pathologies, including malignancies, monogenic diseases, and infectious diseases such as HIV-1. Here we studied whether HSPCs could traffic to reservoir sites, engraft, and give rise to HSPC-derived CNS cells such as microglia. **Methods** Following myeloablative conditioning, pigtail macaque ID J02370 was transplanted with autologous lentiviral vector-modified cells expressing GFP and the P140K mutant of methylguanine methyltransferase ($MGMT^{P140K}$), and followed for almost 10 years. At necropsy, an extensive panel of tissues was collected, and GFP marking in hematopoietic subsets was measured by flow cytometry. Gene-marked, tissue-associated macrophages were classified on the basis of CD68, CD11b, CD11c, and/or CD206 expression. Functionally phagocytic macrophages were identified via uptake of fluorescent bead substrates. CNS tissues were isolated and characterized by flow, immunohistochemistry, and retrovirus integration site (IS) analyses. CNS samples from a cohort of three additional transplanted macaques were collected and analyzed for comparison. **Results** We observed the highest proportion of gene-marked cells in secondary lymphoid tissues including lymph nodes and spleen, as well

as liver, gastrointestinal tract, and reproductive tissues. Phenotyping of lymphoid and myeloid populations demonstrates that gene-marked cells are present without subset bias. We observe efficient marking of multiple macrophage subsets, suggesting that HSPC gene therapy is a viable approach to target gene-modified myeloid cells to tissues. Importantly, we also observe gene marking in CNS-associated microglia cells. IS analyses demonstrate that gene-marked cells in the CNS from J02370 are compartmentalized and unique from gene-marked cells at any other physiological site, including peripheral blood. IS in a comparable cohort of 3 animals reinforce our finding that CNS-compartmentalized, gene-modified HSPCs seed unique progeny in the brain. **Conclusions** Although the ability of HSPC-derived cells to traffic to secondary lymphoid tissues such as spleen and lymph nodes is well known, the engraftment of HSPCs and their progeny in CNS is less understood. Here, we leveraged our nonhuman primate model of HSPC transplantation to quantify the trafficking and engraftment of marked cells in over 20 tissue compartments, including HIV reservoir sites such as CNS. These results demonstrate the promise of HSPC gene therapy for the delivery of therapies to the CNS, namely for the elimination of latent HIV reservoirs, as well as for various malignant and genetic diseases with associated CNS pathologies.

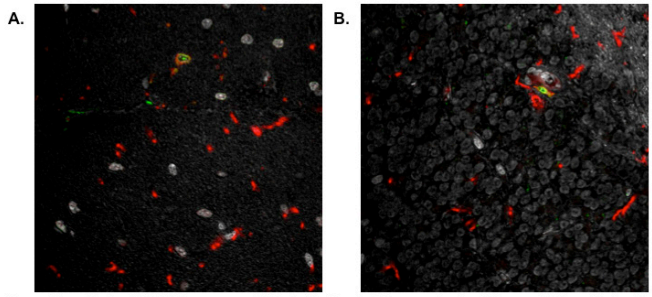

Figure: Gene-Marked HSPC Progeny are Detected in Central Nervous System Tissues. GFP-modified cells *in situ* following HSPC transplantation of animal ID J02370. Shown are A) molecular layer, and B) granular layer from cerebellum following immunofluorescence staining with antibodies against GFP (green) and the microglia marker Iba1 (red); double positive cells are yellow. Gray indicates DAPI staining for nuclei.

442. Turning GWAS into Treatments: Allele-Specific Functional Modeling of the Multi-Cancer Risk Locus 8q24 via CRISPR/Cas9

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In the past decade, Genome-Wide Association Studies have identified a large number of genomic variants, specifically single nucleotide polymorphisms (SNPs), associated with increased cancer risk. The molecular mechanisms underlying the vast majority of these associations remain largely unknown. Precise cellular models containing the variant of interest could be a crucial tool for establishing disease-relevant function in order to translate these discoveries into clinical application. CRISPR/Cas9 gene editing has provided a convenient and flexible method for creating isogenic cell lines via the homology-directed repair (HDR) pathway, however methods for efficient generation and subsequent isolation of precisely edited cells have proven both expensive and time-consuming. In response to this need, we have combined CRISPR/Cas9 HDR gene editing with an innovative high-throughput genotyping pipeline utilizing KASP (**K**ompetitive **A**llele-**S**pecific **P**CR) technology to create scarless isogenic cell models of cancer risk variants in ~1 month without selectable markers or specialized methods such as digital droplet PCR or NGS. Utilizing this technology, we have been able to create cell lines differing by only a single base to model risk-associated SNPs located in the 8q24 risk locus in multiple cell types. The 8q24 risk locus is associated with increased risk for colorectal, breast, prostate, and more recently thyroid cancer. In this study, we use engineered isogenic cell lines modeling functional risk SNP rs6983267 in each of these cancer types to identify tissue-specific mechanisms of this multi-cancer risk locus. Rs6983267 is located within a known c-Myc enhancer and has been shown to exert risk-allele specific increase in enhancer activity. Oncogenic c-Myc is known to play a significant role in the pathogenesis of many cancers. However, how or if this mechanism varies between the different cancer this locus has been associated with remains uncertain. Utilizing our novel assay, ChIPnQASO, to detect allelic imbalances of transcription factor binding at heterozygous loci, we do observe cell type variation in the regulatory effect of rs6983267. Not only does there seem to be a varying effect of the risk allele on c-Myc expression across the different cell types, but also risk allele enhancement of binding of the TCF7L2 transcription factor to the c-Myc enhancer rs6983267 is located within. Interestingly, rs6983267's regulatory effect seems most significant between the homozygous reference and heterozygous genotypes within the same tissue type. We are delving further into these differences using RNA-Seq and allele-specific 4C-Seq to assess whole transcriptomic and chromosomal interaction changes with changes in risk status. In addition, we plan to complete mouse model studies with injection of our engineered cancer cells in order to identify any effects on tumorigenesis *in vivo* due to the risk status of injected cells. This study is one of first to compare risk mechanisms across different cancer tissue types; using precisely edited cellular models, we are able to detect cell-type specific variations in functional risk SNP effects on cancer development. The results of this study will allow for initial steps towards precision medicine, utilizing germline variation knowledge to inform disease treatment and even prevention in an individual level.

443. Virus Vector-Mediated Genetic Modification of Brain Tumor Stromal Cells after Intravenous Delivery

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The malignant primary brain tumor, glioblastoma (GBM) is generally incurable. New approaches are desperately needed. Adeno-associated virus (AAV) vector-mediated delivery of anti-tumor transgenes is a promising strategy, however direct injection leads to focal transgene spread in tumor and rapid tumor division dilutes out the extra-chromosomal AAV genome, limiting duration of transgene expression. Intravenous (IV) injection gives widespread distribution of AAV in normal brain, however poor transgene expression in tumor, and high expression in non-target cells which may lead to low therapy and high toxicity, respectively. Delivery of transgenes encoding secreted,

anti-tumor proteins to tumor stromal cells may provide a more stable and localized reservoir of therapy as they are more differentiated than fast-dividing tumor cells. Reactive astrocytes and tumor-associated macrophage/microglia (TAMs) are stromal cells that comprise a large portion of the tumor mass and are associated with tumorigenesis, and would provide a reasonable target for AAV. However, existing data suggests that myeloid derived cells such as microglia are recalcitrant to AAV transduction. In mouse models of GBM, we used IV delivery of exosome-associated AAV vectors driving green fluorescent protein expression by specific promoters (NF-kB-responsive promoter and a truncated glial fibrillary acidic protein promoter), to obtain targeted transduction of TAMs and reactive astrocytes, respectively, while avoiding transgene expression in the periphery. We used our approach to express the potent, yet toxic anti-tumor cytokine, interferon beta, in tumor stroma of a mouse model of GBM, and achieved a modest, yet significant enhancement in survival compared to controls. Currently we are combining AAV-mediated tumor stromal cell expression of interferon beta with the standard of care drug, temozolomide, whose effects are known to be sensitized by interferon beta.

444. Implantable Scaffolds Enhance Neural Stem Cell Therapy for Resected Glioblastoma

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Introduction: Glioblastoma (GBM) is a devastating brain cancer with a dismal 5-year survival rate of only 5%, due in large part to the invasiveness of individual GBM cells. Tumoricidal neural stem cells (tNSCs) inherently chase cancer cells, making them ideal vehicles to deliver GBM therapies. To remain therapeutically effective, transplanted tNSCs must persist in the brain despite inhospitable postoperative conditions caused by standard-of-care surgical resection. In this study, we aim to demonstrate, via mouse models incorporating fluorescence-guided surgical resection, that tNSCs are made more effective against highly invasive GBM when delivered into the brain on supportive implantable scaffolds. **Methods:** Lentiviral constructs were used to induce stable expression of fluorescent and bioluminescent markers for *in vitro* and *in vivo* tracking of NSC and GBM cell populations. For *in vitro* studies, we examined the attachment, growth, stemness, and tumoritropic migration of human HB1.F3 tNSCs on either poly(lactic acid) (PLA) or gelatin (GEM) scaffolds. For *in vivo* testing, we created surgical resection cavities in the brains of nude mice and then implanted scaffolds bearing tNSCs into the resection cavities. Bioluminescence imaging (BLI) was used to track transplanted cell persistence. *In vivo* efficacy studies were conducted by xenografting and later surgically resecting patient-derived human GBM8 tumors in nude mice. tNSCs were engineered to express thymidine kinase ($tNSC^{tk}$), an enzyme which converts the prodrug ganciclovir (GCV) into cytotoxic ganciclovir triphosphate (GCV-TP), and seeded onto GEM ($GEM/tNSC^{tk}$). $GEM/tNSC^{tk}$ were implanted into the resection cavity at the time of surgery. Therapy was initiated in the treatment group three days later via daily intra-peritoneal injections of 100 mg/kg GCV and continued for 14 days, while the control group (GEM/NSC^{tk}) received saline injections. Tumor progression was tracked via bi-weekly BLI. Tissues and scaffolds were collected upon study completion and analyzed for cell content. **Results:** *In vitro* tests showed that tNSCs attached, grew, and maintained stemness on both PLA and GEM scaffolds. *In vivo*, PLA provided a modest improvement in the persistence of transplanted tNSCs. Adjustments to surface coating, fiber diameter, and morphology of the PLA did little to further improve persistence. In contrast, GEM increased median NSC persistence 7-fold when compared to direct injection control and remained permissive to tumoritropic homing. Intra-cavity transplant of $GEM/tNSC^{tk}$ significantly suppressed post-operative GBM tumor recurrence and extended median survival in mice from 31 to 46 days (Figure 1).

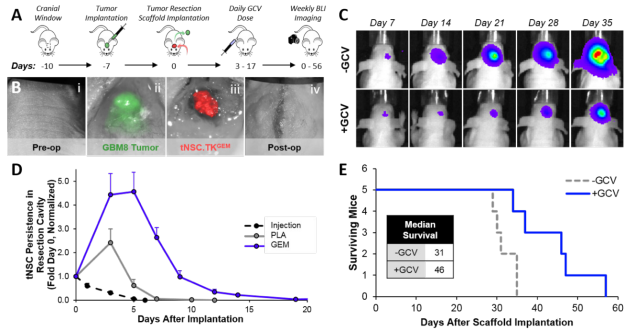

Figure 1: tNSC therapy is more effective on GEM scaffolds. A) Procedural timeline. B) Intraoperative images. C) BLI data. D) Persistence benefit of PLA and GEM compared to injection. E) Kaplan-Meier survival curves. **Conclusions:** Together, these data begin to define the scaffold design parameters required to efficiently transplant tNSCs into the post-operative GBM cavity and may improve the clinical application of tNSC therapy for brain cancer.

445. Precise Anatomical Localization and Deep Tissue Imaging of Cells Expressing Luciferase and the Sodium Iodide Symporter (NIS)

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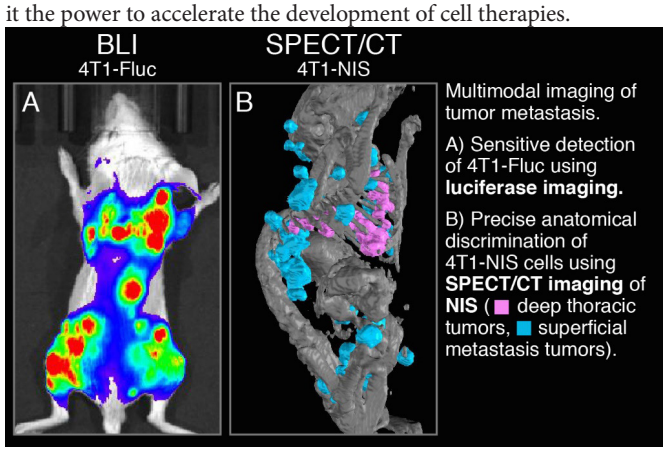
Noninvasive imaging of cells expressing reporter genes provides a powerful tool for understanding cell fate in living animals. High sensitivity and relative ease of use have made bioluminescent imaging (BLI) common in pre-clinical studies. Nonetheless, BLI is limited by poor signal penetration, making it unusable for deep tissue or large animal imaging and making precise anatomical localization of signal difficult. Moreover, its application is strictly limited to pre-clinical studies. Here, we employed a multimodal approach that combined BLI with tomographic SPECT/CT and PET/CT imaging of the sodium iodide symporter (NIS), in order to demonstrate the advantages of combining optical and nuclear reporter gene imaging

for tracking cells in vivo. To this end, we generated a series of tumor models expressing firefly luciferase (Fluc) and NIS. *In vitro*, Fluc and NIS activity exhibited strong linear correlation to cell number over a wide range of cell concentrations. For *in vivo* studies, we implanted syngeneic Balb/c mice with mammary carcinoma 4T1 cells. BLI signal from 4T1-Fluc cells was detected in the lungs early after intravenous implantation and increased rapidly until the mice were euthanized due to tumor burden. Upon autopsy, numerous subcutaneous tumors were observed and correlated strongly with BLI signal. However, little BLI signal was detected in the lungs of the mice, despite the presence of significant lung tumor nodules that exhibited high BLI signal upon removal from the thoracic cavity. Thus, deep tissue BLI signal was masked by overwhelming surface signal from the subcutaneous tumors. In contrast, lung 4T1-NIS tumors were readily distinguished in the living mice by SPECT or PET imaging. Moreover, the NIS signal in these tumors could be accurately quantitated and anatomically localized through combined CT imaging. To further assess the utility of multimodal Fluc and NIS tumor imaging, we implanted mice with lymphoma Nalm6-Fluc-NIS cells and performed BLI and SPECT/CT imaging. Tumors were detected earlier with BLI than SPECT, but on later days NIS signal by SPECT strongly correlated to Fluc signal by BLI. BLI imaging suggested that Nalm6-Fluc-NIS tumors were primarily localized to the spine and long bones. Importantly, SPECT/CT imaging was able to precisely localize the tumors to the bone without autopsy. Together, our data show the advantages of combining BLI and SPECT or PET imaging of NIS for tracking cells in pre-clinical studies. BLI can be used for sensitive early detection and relatively easy monitoring of cell growth, while NIS imaging at later times can be used to achieve superior resolution, deep tissue imaging, quantification, and precise anatomical localization. Thus, NIS imaging provides valuable information about cell fate that cannot be realized by BLI alone, giving it the power to accelerate the development of cell therapies.



446. Selective Killing of Virus-Transduced or Cancer Cells Using a RNA *Trans*-Splicing Based Suicide Gene Therapy Approach

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Acquired and inherited genetic disorders are characterized by the cellular expression of aberrant transcripts and proteins. Viruses such as the human papillomaviruses (HPV) or the human immunodeficiency virus type 1 (HIV-1) integrate their DNA into the genome of the infected host cell and there is no way to get rid of the viral DNA anymore. Similarly, many cancers are characterized by oncogene expression. We explored a Herpes simplex virus thymidine kinase (HSVtk)/ganciclovir (GCV) suicide gene therapy approach for selective killing of virus-transduced or cancer cells. First we studied the highly efficient mechanism of *trans*-splicing among transcripts of the simian virus 40 (SV40). Then we employed molecular features of SV40 RNA *trans*-splicing and computational RNA structure design to improve both on-target activity and specificity of the *trans*-splicing RNA (tsRNA). As molecular targets we selected the α-fetoprotein (AFP), a marker of hepatocellular carcinoma (HCC), human papillomavirus type 16 (HPV-16), or human immunodeficiency virus type 1 (HIV-1) pre-mRNA. While unstructured mismatched target binding domains significantly improved 3' exon replacement, 5' exon replacement correlated with the thermodynamic stability of the tsRNA 3' end. Alternative on-target *trans*-splicing was found to be a prevalent event. The specificity of *trans*-splicing with the intended target splice site was improved 10-fold by designing tsRNA harboring multiple target binding domains shielding alternative on-target and blinding non-target splicing events. Rationally designed tsRNAs efficiently and selectively triggered death of HPV-16, HIV-1 or AFP-positive cells. Dual-targeting tsRNA simultaneously targeting AFP and a second HCC biomarker triggered enhanced cell death at 10-fold lower GCV doses. With regard to delivery, novel dumbbell-shaped DNA minimal vectors were found to be more potent than conventional plasmids. Currently we explore targeting liver cancer patient-derived xenografts in three-dimensional spheroids and an orthotopic mouse model. Our observations suggest RNA *trans*-splicing represents a promising approach to suicide gene therapy targeting viral infection, cancer or other diseases characterized by the expression of one or multiple disease-specific pre-mRNA biomarkers.

References

Jiang X, Yu H, Teo CR, Tan G, Goh SC, Patel P, Chua YK, Hameed NBS, Bertoletti B, Patzel V*. Advanced design of dumbbell-shaped genetic minimal vectors improves non-coding and coding RNA expression. *Molecular Therapy* 24(9), 1581-1591, 2016.
Ingemarsdotter CK, Poddar S, Mercier S, Patzel V, Lever AML*. Expression of herpes simplex virus thymidine kinase/ganciclovir (HSV-tk/GCV) by *trans*-splicing induces selective killing of HIV producing cells. *Molecular Therapy Nucleic Acids* 7, 140-154, 2017.
Poddar S, Loh PS, Ooi ZH, Osman F, Eul J, Patzel V*. RNA structure

design improves activity and specificity of *trans*-splicing triggered cell death in a suicide gene therapy approach. *In press at Molecular Therapy Nucleic Acids.*

447. Dual Prodrug Activator Gene Therapy with Pseudotyped Retroviral Replicating Vectors

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Introduction: Retroviral replicating vectors (RRV) exhibit tumor-selective replication and achieve stable integration into the cancer cell genome, enabling continued virus production and intratumoral gene transfer over time. RRV engineered with prodrug activator ('suicide') genes have shown promising results in preclinical studies and early phase clinical trials, and this strategy is being evaluated in an international Phase III clinical trial for recurrent high-grade glioma. The aim of the current study was to evaluate feasibility and efficacy of using two RRVs encoated ('pseudotyped') with different envelope proteins in order to overcome superinfection resistance in cancer cells, and thereby enable combination therapy with two different prodrug activator genes. **Methods**: Replication of amphotropic murine leukemia virus (AMLV) envelope- and gibbon ape leukemia virus (GALV) envelope-pseudotyped RRV, either individually or in combination, was monitored art least 27 days in established and primary human ovarian cancer cell lines (SKOV3ip, OVCAR-5, OCI-P5X, OCI-C5X) at various multiplicities of infection (MOI) ranging from 1 - 0.001. Flow cytometry was used to determine transduction levels, by detecting expression of fluorescent protein (GFP or mStrawberry) reporter genes delivered by RRV, at serial time points. Additionally, two different prodrug activator genes (yeast cytosine deaminase (yCD) and E. coli nitroreductase (NTR)) were engineered into AMLV- or GALV-pseudotyped RRV and evaluated by MTS assay for their ability to induce cytotoxicity, individually and in combination, upon exposure to different concentrations of their respective prodrugs, 5-fluorocytosine (5-FC) and CB1954. To determine viral integration and vector stability, PCR of genomic DNA extracted from transduced cells was performed using RRV-specific primers, and qRT-PCR was performed to determine vector copy number per cell. **Results**: All human cancer cell lines tested could be > 80% transduced by AMLV- and GALV-pseudotyped RRV, both individually and in combination, within 9-27 days after initial inoculation at MOI as low as 0.001, with only minor differences in replication kinetics between established and primary cancer cell lines. Both pseudotypes could mediate efficient RRV entry and stable integration into the target cell genome without evidence of transgene deletion. Furthermore, MTS cytotoxicity assays demonstrated that RRV-mediated prodrug activator gene therapy is an effective approach for killing ovarian cancer cells *in vitro*. After dual transduction with ALMV- and GALV-pseudotyped RRV expressing yCD or NTR, all cancer cell lines tested showed ≥50 %reduction in viability within 2-4 days at individual prodrug concentrations of 1mM 5-FC and 10μM CB1954, respectively. At optimal concentrations, combination prodrug activator gene therapy with both prodrugs (ranging from 1μM - 10mM 5-FC combined with 10nM - 100μM CB1954) resulted in synergistic cytotoxicity as compared to treatment with either prodrug alone in dually transduced target cells. **Conclusion**: Combining RRV pseudotyped by alternative envelopes can effectively overcome superinfection resistance, enabling dual transduction and synergistic cytotoxicity in cancer cells *in vitro*.

448. Targeting P53 with Pro-Apoptotic Factor Bad to the Mitochondria for Ovarian Cancer Gene Therapy

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Although the main function of p53 is a nuclear transcription factor, p53 can directly trigger the intrinsic apoptotic pathway through the mitochondria. p53 binds to mitochondrial anti-apoptotic proteins (Mcl-1, Bcl-2 and Bcl-XL) and pro-apoptotic effector proteins (Bak and Bax). Once p53 localizes to the mitochondria, it can induce the release of Bak and Bax from anti-apoptotic factors and triggers the oligomerization of Bak and Bax, leading to caspase cascade activation. Targeting p53 to the mitochondria is an attractive approach because it can cause a rapid apoptotic response and bypass the cell cycle arrest pathway. However, the innate mitochondrial localization ability of p53 is too weak for therapeutic relevance. Moreover, the apoptotic effect of p53 alone may not be sufficient to induce apoptosis in all cancers because many cancer cells, especially drug-resistant ones, are known to have high expression of mitochondrial anti-apoptotic factors in the Bcl-2 family. To overcome these issues, we will take advantage of the BH3-only pro-apoptotic proteins, which are potent inhibitors of pro-survival Bcl-2 proteins. Therefore, we hypothesize that p53 can be targeted to mitochondria by attaching the pro-apoptotic factor BAD to the C-terminus of p53 (called p53-Bad), and this p53-Bad will have superior apoptotic activity than wild type p53 with attributes of both p53 and the pro-apoptotic BH3 domain of Bad. Our goal is to use these chimeric constructs as gene therapy for treatment of high grade serous ovarian cancer, where p53 mutation and deletion rate are 96%, and wild type p53 gene therapy is known to ineffective due to the dominant negative inhibition by endogenous mutant p53. The localization of Bad to the mitochondria is controlled by phosphorylation. Bad can localize to the mitochondria using a mitochondrial targeting sequence at the C-terminus, only when Bad is not phosphorylated at Ser112 and Ser136. When these two amino acids are phosphorylated, Bad is sequestered by scaffold protein 14-3-3 and remains in the cytoplasm. We control the localization of p53-Bad by making serine to alanine mutations at residues 112 and 136 of Bad (called p53-Bad/112,136). In Skov-3 cells, p53-Bad/112,136 and the negative control Bad/112,136 (no p53) are effectively localized to the mitochondria, while p53-Bad partially localizes to both the mitochondria and the nucleus. Bad alone remains in the cytoplasm. Nuclear transcriptional activity assays in Skov-3, ID8, Kuramochi, and Ovcar-3 ovarian cancer lines corroborate and show no activity. Multiple assays that represent different stages of cellular apoptosis, including TMRE (mitochondrial outer membrane depolarization), caspase 3/7 (early apoptosis), and 7-AAD assay (late stage apoptosis) were used to compare the apoptotic activity of p53-Bad constructs to p53-wt and the respective negative controls. Our preliminary data indicates that p53-Bad and p53-Bad/112,136 have superior activity over p53-wt in all

ovarian cell lines tested so far, regardless of the p53 status. The activity of p53-Bad/112,136 is more than 7-fold higher than p53-wt in terms of caspase activation in Skov-3 and 4 times higher than p53-wt in ID8 and Ovcar-3 as detected by mitochondrial potential depolarization. Our next step is to test our constructs in vivo in a syngeneic orthotopic metastatic ovarian cancer mouse model.

449. Development of a Reproducible and Scalable Method for Manufacturing *p*DNA//PEI Nanoparticles as a Plasmid Delivery Agent

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Plasmid DNA (*p*DNA) nanoparticles synthesized by linear polyethylenimine (*l*PEI) have shown to be effective gene delivery vehicles for therapeutic applications in animal models. However, the majority of these developments have failed, either in late-stage preclinical or in early-stage clinical studies, largely due to challenges in formulation optimization, batch-to-batch reproducibility, scalable manufacturing, and reliable characterization and screening methods. We have developed a scalable, reproducible, and controllable manufacturing method for production of shelf-stable *p*DNA/*l*PEI nanoparticles. Specifically, we have turned a batch mode (bulk mixing) method into a continuous process using the flash nanocomplexation (FNC) method [1]. We have demonstrated excellent reproducibility for this FNC process under quality assurance and quality control standards at different production batch sizes. The sizes of the FNC *p*DNA/*l*PEI nanoparticles were tunable with a low PDI, and the zeta potential ranged from 30 to 50 mV. In addition, the FNC nanoparticles could be reliably lyophilized. The lyophilized formulation preserved the composition, physicochemical properties, and bioactivity of the nanoparticles; and, to date, a stability for up to 6 months in -20°C has been achieved. Furthermore, systemically delivered FNC nanoparticles were well-tolerated in mice and a large animal model, and biodistribution, determined by bioluminescence and QPCR respectively, showed delivery of the plasmids to many organs/tissues. These results confirmed that FNC production could overcome many manufacturing limitations of *p*DNA/*l*PEI nanoparticles and open up new opportunities for clinical translation of DNA nanomedicine products.

Reference: [1] J. L. Santos et al., "Continuous Production of Discrete Plasmid DNA-Polycation Nanoparticles Using Flash Nanocomplexation", Small, 12 (45): 6214-6222 (2016).

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450. Tumor Activity of Candidate Promoters in Canine Tumors

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Expression targeting in cancer gene therapy utilizes tumor upregulated expression of promoters to govern expression of genes, such as therapeutic genes or, in oncolytic viruses, genes controlling replication. This results in tumor specific expression with reduced toxicity in normal cells. Although several promoters have been tested for specificity in a variety of human cancers, few have been evaluated for canine tumors. The dog is considered as an outstanding animal model for human cancer as it possesses characteristics such as inter-individual and intratumoral heterogeneity and genomic sequence instability similar to human tumors. Thus, our goal was to identify and investigate the tumor activity of canine survivin (cSurvivin), canine chemokine receptor 4 (cCXCR4) and canine telomerase reverse transcriptase (cTERT) as they showed high levels of tumor-specific expression in a variety of human cancers and murine models. In addition, we also investigated the tumor expression of an exogenous E2F modified E1a promoter (E2F-E1a) of canine adenovirus type 2. To accomplish this goal, we evaluated the activity of the endogenous promoters in canine hematopoietic and non-hematopoietic (canine B- and T cell lymphoma) tumor cells and tissues by employing Q-RT-PCR and by measuring GFP reporter gene expression level to evaluate exogenous promoter activity. Results showed negligible endogenous expression differences between canine normal and both hematopoietic and non-hematopoietic tumor cells for cTERT promoter, although it showed increased tumor-specificity in the human and mouse model as proved by several previous studies. However, cSurvivin showed markedly higher endogenous activity in both hematopoietic and non-hematopoietic tumor cells/tissues with reduced expression in most normal cells and tissues. Although, cCXCR4 showed reduced endogenous activity for most of the canine tumors/ tissues, it showed high levels of activity for canine T-lymphoma cells and tissues. To further validate these findings, we cloned the sequences of these promoters in GFP reporter plasmids to measure exogenous promoter activity using a series of cellular transfections and co-transfection experiments (CMV-tdTomato as internal control) followed by measuring reporter gene expression through flow cytometry normalized to CMV expression. When both the reporter and normalization plasmids were co-transfected, the CMV promoter interfered with the activity of all the tested promoter, resulting in lower levels of expression of these promoters. Although results were congruent with RT-qPCR data for cTERT, cSurvivin, cCXCR4 and E2F-E1a showed high level of canine tumor-specific expression than that of normal cells. However, individual promoter activity was not uniform for all types of cancers; rather it varies based on tumor types. These findings imply that identification of a pan-cancer promoter may be difficult and that expression targeting may need to rely on precision medicine approaches to select patient-specific promoters to drive the activity of therapeutic gene in a patient-specific manner.

451. Folic Acid Conjugated Chitosan-Functionalized Gold Nanoparticles for Targeted Delivery of 5-Fluorouracil in Breast Cancer

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Optimization of the pharmacological potential of a drug with reduction in the adverse effects is the major prerequisite for a drug delivery system. The use of functionalized nanoparticles (NP) can provide site specific and targeted delivery along with optimal drug release. The use of gold nanoparticles (GNPs) has increasingly become popular owing to its inherent optical features, relative non-toxicity and suitable biocompatibility. Chitosan (CS) is a non-toxic biocompatible polymer that can effectively functionalize and stabilize GNPs in an aqueous solution. 5-fluorouracil (5-FU), a thymidylate synthase inhibitor, is extensively used in the treatment of solid tumours. However, poor bioavailability, short plasma half-life coupled with off-target cytotoxicity has limited its use in chemotherapy. To improve the therapeutic efficacy of 5-FU with minimal side effects, it can be selectively delivered to target sites using specifically designed carriers. Further, the folate receptor which is highly expressed in various human cancers can be targeted for receptor-mediated uptake of chemotherapeutics. Folic acid is widely used as a ligand for folate receptor because they are cheap, nontoxic, retain high affinity for folate receptors, and are stable in the systemic circulation. In the present study, 5-FU loaded gold nanoparticle (GNP) was functionalized and stabilized with Folic acid (FA) conjugated Chitosan (CS) (FA-CS-GNP-5-FU) for improved drug efficacy with minimal side effects. Drug-excipient interaction was achieved by ionic cross linking mechanism and drug encapsulation efficiency by the nanoparticles (NPs) was appropriately determined. The physiochemical properties of the NPs were investigated using UV-visible spectroscopy, transmission electron microscopy (TEM), attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) and zeta/nanoparticle tracking analyser. Also, *In vitro* drug release study was done by simple dialysis method and *in vitro* cytotoxicity was evaluated on human breast adenocarcinoma cells (MCF-7) using MTT and dual acridine orange/ethidium bromide apoptosis assays. FA-CS-GNP-5-FU, which were spherically shaped with an average size of approximately 31nm, displayed surface Plasmon resonance bands (SPR) at 525nm, confirming the synthesis of small-sized GNPs. FTIR analysis showed the presence of 5-FU and FA-CS on the nanocomposite. FA-CS-GNP-5-FU was highly stable in aqueous medium as revealed by its hydrodynamic zeta potential value of 57.9mV. The targeted carrier which had drug encapsulation efficiency of over 70%, presented a pH dependent sustained release of 5-FU. Drug release kinetics suggests controlled diffusion as the mechanism of drug release from the delivery vehicle. Furthermore, FA-CS-GNP-5-FU exhibited tumour specific cytotoxicity with a higher efficacy as compared to free 5-FU. The half maximal inhibitory (IC_{50}) value for FA-CS-GNP-5-FU on MCF-7 cells was approximately 20 µg/mL while free 5-FU at an identical dose resulted in less than 30% cell death. Therefore, it is conceivable that the improvement in the efficacy of 5-FU would bring about decreasing the dosage regimen, thus, minimizing the adverse effects usually caused by the repeated administration of 5-FU. The presence of FA on the nanocomposite induced specific binding to the folate receptors (FRα) which are highly expressed by MCF-7 cells. The interaction facilitated folate receptor mediated endocytosis, resulting in enhanced uptake of FA-CS-GNP-5-FU by the MCF-7 cells. An unequivocal proof to this claim was obtained in a competition assay. It can therefore be concluded that FA-CS-GNP-5-FU is a promising therapeutic system for cancer treatment.

452. Cystic Fibrosis Transmembrane Conductance Regulator is a Tumor Suppressor in Non-Small Cell Lung Cancer via Targeting Wnt/Beta-Catentin Signaling

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China Lung cancer remains a leading cause of cancer-related deaths worldwide. The cystic fibrosis transmembrane conductance regulator (CFTR) plays a crucial role in retaining the homeostasis and function of lung, its expression and function were found to correlate with many types of epithelial cancers including the lung cancer. An impaired CFTR function or loss of CFTR protein might increase the risk of development of lung cancer. Wnt/β-catenin signaling governs the cell fate and proliferation in a variety of cell types, and prominently features in stem cells and cancers. Interestingly, the abundance of CFTR was inversely correlated with Wnt/beta-catenin signaling activity. In this study, the expression of CFTR in smokers and non-smokers of lung adenocarcinoma, and the impact of interactions between CFTR and Wnt/beta-catenin signaling on the metastatic property of lung cancer A549 cells were examined using assays of cell migration, invasion and clonogenicity, as well as molecular analysis of the expression of lung cancer stem cell (LCSC) markers and interaction of proteins by cytometry, immunoblotting and immunoprecipitation assays. Remarkably, the expression of CFTR was significantly down-regulated in lungs of smokers and nicotine-treated A549 cells. In vitro results further demonstrated that an overexpression and knockdown of CFTR expression respectively inhibited and enhanced the capacities of cell migration, invasion and clonogenicity, as well as the frequency of ALDH positive cells in both A549 cells treated with and with nicotine. Molecular analysis demonstrated that CFTR-altered LCSC properties were inversely correlated with Wnt signaling activity, and the expression of stem cell markers OCT4, SOX2 but not ALDH1. In contrast, an activated and inactivated Wnt/beta-catenin signaling led a suppression and argumentation of CFTR expression, along with an inhibited and enhanced capacity of LCSCs, respectively. Immunoprecipitation assay further revealed interactions of CFTR and Wnt/beta-catenin signaling APC, Dishevelled 2 and beta-catenin. This study thus provides a novel mechanism by which CFTR modulates Wnt/beta-catenin signaling to reduce metastatic properties and indicates a potential therapeutic target for lung cancer.

453. IL13Rα2-CAR T Cells Persist for Up to 21 Days after Systemic Administration in Mouse Glioma Models

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Glioblastoma Multiforme (GBM) is the most common and aggressive brain tumor. GBM is untreatable due to its highly infiltrative nature and resistance to current therapies. New therapeutic strategies are urgently needed to kill tumor cells precisely while minimizing damage to the neighboring normal brain tissue. CAR therapies hold promise in the treatment of GBM without the usual toxicities associated with cytotoxic regimens. In a clinical setting effective therapy requires that systemically administered CAR-T cells migrate to tumor cells and persist within the tumors. We have developed and preclinically tested *novel* IL13Rα2-CAR T cells. We have proved that in vivo systemic delivery of IL13Rα2-CAR T cells as a single agent delays tumor growth, prolongs survival, and that IL13Rα2-CAR T cells persist within the mouse brain for up to 21 days. These data provide evidence for an effective therapy of the treatment of GBM.

454. Xrcc1 or Mthfr Gene: Superior Gene for Predicting a Response during Folfox Chemotherapy for Metastatic Colorectal Cancer

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Background: While there are immense interests in treating cancer, the biggest method that is expected to be the biggest issue of the colon cancer treatment is tailored chemotherapy based on drug sensitivity and pharmacogenetics. In case of metastatic colon cancer, the combination of 5-flurouracial and folinic acid (FOLFOX) and oxaliplatin was proved to work well on the patients. Studies have shown that there are several genes that suspend the effects of 5-FU and oxaliplatin but it is not clear which gene is the dominant gene at this time. **Objectives:** The main objective of this study was to figure out, in FOLFOX chemotherapy, which gene polymorphism is a dominant factor; x-ray cross-complementing 1 (XRCC1) gene for oxaliplatin and methylenetetrahydrofolate reductase (MTHFR) gene for 5-fluorouracil. **Patients and Methods:** Total of 54 patients, who have unresectable distant metastasis from colon cancer, were gathered for this study. These patients went through chemotherapy with FOLFOX regimen. Facilitated with a polymerase chain reaction-based fluorescence based direct sequencing, two genes that were from paraffin-embedded tissues, which were MTHFR polymorphisms in XRCC1 gene (Arg/Gln substitution in exon 10) and MTHFR gene (C677T, alanine valine mutation), got analyzed. Depending on types of polymorphism, survivals and response rates were compared. **Results:** In MTHFR polymorphism, 16 patients (32.0%) showed no mutation (Ala/Ala), 28(56.0%) mutations in one allele (Ala/Val) and 6(12.0%) mutations in both alleles (Val/Val). In XRCC1 polymorphism 31 patients (60.8%) showed no mutation (Arg/Arg), 16(31.4%) mutations in one allele (Arg/Gln) and 4(7.8%) mutations in both alleles (Gln/Gln). On completion of 4 cycles of FOLFOX, one patient (1.9%) showed complete remission, 31(57.4%) showed partial remission or stable disease, and 21(40.7%) showed disease progression. MTHFR polymorphism was not significant in predicting response (P=0.234) and 30 months-survival (P=0.418), whereas XRCC1 polymorphism was a significant prognostic factor for both response (P=0.021) and survival (P=0.022). **Conclusions:** In Korean colon cancer patients, the study showed that the rate of mutation in MTHFR gene (68%) was higher than the rate of mutation in MRCC1 gene (39%). As for MTHFR's mutated group, the response rate came out to be higher while the response rate as lower in XRCC1's mutated group. But, for the response to FOLFOX chemotherapy and survival, only the XRCC1 polymorphism is a significant prognostic factor.

455. Effects of Acid Ceramidase Expression on Cortisol Secretion in H295R Adrenal Carcinoma Cells

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The enzyme acid ceramidase has been shown to be over-expressed in some cancer cell lines and primary tumors, and enhances the ability of these cells to convert ceramide, which is often produced as a pro-apoptotic response to stress, to sphingosine, which can then be converted to the pro-survival molecule sphingosine-1-phosphate. Therefore, acid ceramidase over-expression confers a survival advantage in response to stress such as chemotherapy and radiation. Cortisol is a steroid stress hormone produced by adrenal cells in response to adrenocorticotropic hormone released by the pituitary gland. Published studies have shown that adrenocorticotropic hormone can also increase acid ceramidase expression. In order to evaluate the effects of increased acid ceramidase expression on adrenal cells, H295R adrenal carcinoma cells were transfected to create stable lines over-expressing acid ceramidase. As previously reported for other cell types, increased acid ceramidase expression decreased cellular sensitivity to ceramide treatment. These cells also had increased production of cortisol compared to a control cell line, suggesting that AC expression may influence cortisol synthesis. Acid ceramidase expression in these cells decreased over time while growth in selection media was unaltered, likely due to silencing of the CMV promoter. This study describes the construction of a new acid ceramidase expression plasmid using the EF-1α promoter and characterization of H295R cells transfected with this plasmid.

Cell Therapies II

456. Abstract Withdrawn

457. Expression of a TIM8 Peptide Reduces Alloreactivity of T Cells Facilitating an Allogeneic NKG2D Chimeric Antigen Receptor T Cell Therapy Approach

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T cell recognition of antigens occurs through the T cell receptor (TCR) and is dependent on recognition of peptides in the context human leukocyte antigens (HLAs) on the surface of target cells (such as antigen presenting cells). Many tumors have developed escape mechanisms from this immune recognition. Chimeric-antigen receptor (CAR) modified T cells offer an alternative to conventional TCR stimulation by the circumvention of the TCR mediated activation and allow the recognition of a specific antigen. Most CAR T cell therapy relies on autologous cells, in which the treatment consists of cells from the patient themselves. Alternatively, cells derived from an allogeneic donor could be used. However, allogeneic cells face two problems: graft versus host (GvH) and host versus graft (HvG) responses. The HvG response means that host cells would eliminate the allogeneic grafted cells from the donor therefore limiting the persistence of the transferred cells. Allogeneic cells can also potentially induce a GvH response and trigger GvH disease (GvHD). These responses are triggered by the recognition of non-self HLA molecules expressed on recipient cells by the TCR of donor cells, therefore leading to potential safety issues for the recipient. In order to avoid GVHD, we targeted the TCR signalling of CAR T cells by different approaches. The most promising strategy was a peptide consisting of a truncated form of CD3ζ called TIMs (TIM - TCR inhibitory molecule). TIM T cells showed a significant reduction in CD3 mediated stimulation and subsequent cytokine secretion when compared to Mock transduced T cells. Furthermore, mixed lymphocyte responses were also lower in the TIM group in comparison to the Mock transduced group. *In vivo* models assessing GvHD showed no GvHD development in a number of mice treated with TIM transduced T cells in contrast to Mock transduced T cells, which were first screened based on *in vitro* CD3 activation. To assess whether TIM could successfully be used in the context of a CAR therapy, a new CAR T cell was developed coexpressing TIM and an NKG2D specific CAR. This allogenic NKG2D CAR, also called CYAD-101, efficiently delayed tumor progression and increase survival in NSG mice bearing orthotopic colorectal tumor. Preliminary results to understand the mode of action of TIM8 showed that TIM8 was acting as a dominant negative form of CD3ζ therefore inhibiting different actors of the TCR signaling pathway like ZAP70 or Erk. These promising results show that the TIM construct does not interfere with NKG2D-CAR mediated anti-tumor activity while inhibiting GvHD, offering an attractive allogenic CAR T cell therapy. The clinical development of CYAD-101 will be further pursued in a phase I trial to assess the safety, the cell kinetic and clinical activity of multiple doses administered concurrently with a standard chemotherapy in patients with unresectable metastatic colorectal cancer.

458. Aggregation of MSCs Leads to a Shift in Secretome Due to Activation of an Ep Receptor-PGE2 Pathway

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Introduction: Upon local injection *in vivo*, MSCs form 3D aggregate structures, which exhibit vastly different gene expression profiles compared to 2D cultured MSCs. These differences lead to significant changes in cell behavior *in vivo* compared to what would be predicted by *in vitro* experiments with 2D cultured cells. Understanding the MSC post-transplant spheroid phenotype is critical to ensuring MSC function and consistency as a cell therapy. Upon aggregate formation, transcriptional changes lead to changes in secreted molecules, cell surface receptors, extracellular matrix molecules, and transcription factors. One of the largest changes is in prostaglandin E2 (PGE2), a small molecule synthesized by COX-2 and PGE synthase which is significantly upregulated in spheroids. PGE2 has four receptors (EP1-4), the expression which dictates the effect of PGE2 signaling on cell phenotype. Despite, spheroid MSCs producing high levels of PGE2 and expressing EP1-4, little is known about how MSCs regulate their EP receptors or the role autocrine signaling through the PGE2-EP pathway plays in controlling MSC phenotype. **Methods:** We used *in vitro* MSC spheroids, formed through a hanging droplet method, as a way to mimic *in vivo* aggregation that occurs after local injections of concentrated cell suspensions (Figure 1A,B). This allowed for detailed analysis of the transcriptional and secretory changes that occur upon MSC aggregation into spheroids and examination of the mechanisms that control the shift in MSC phenotype. **Results:** We have made the novel discovery that PGE2 production increased over 30-fold in spheroids and transcripts for the PGE2 receptors EP2 and EP4, increased by over 20-fold and 3-fold respectively in spheroids (Figure 1C). Blockade of the PGE2-EP2 pathway within spheroids using an EP2 antagonist and/or COX-2 inhibitor led to downregulated expression of EP1, EP2, EP3, and PTGS2 (COX-2) genes (Figure 1D). To determine if signaling factors produced by spheroids induced the phenotypic switch between the 2D and spheroid MSCs, we performed a series of inhibitor and add-back studies. We found that the addition of spheroid secreted products such as IL-1β and PGE2 or antagonism of the cAMP signaling pathway alone did not recapitulate the changes in spheroid MSC EP receptor expression in 2D cultured cells. While the spheroid phenotype is stable over seven days and produces high expression of VEGF, HGF, and TGF-β, the phenotype rapidly reverts to that of 2D cells when spheroid MSCs are disassociated and plated on tissue culture plastic over 1, 3, and 5 days (Figure 1E). Collectively this provides evidence that spheroid formation induces an autocrine feedback loop in MSCs and that feedback loop is initiated by the 3D structure of the spheroid and not single soluble factors alone.

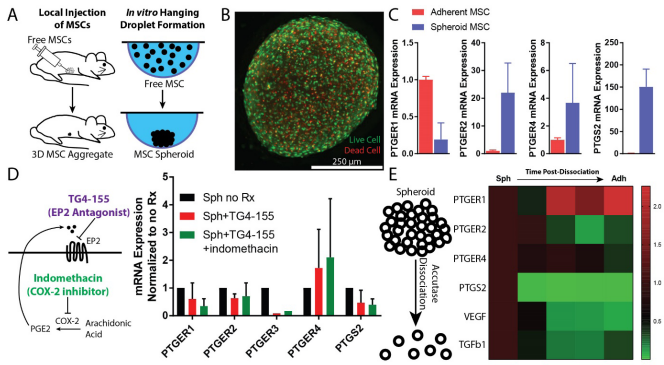

Figure 1: *In vitro* MSC spheroid aggregation leads to upregulation of PGE2 receptors 2/4 to detect synthesized PGE2.

Conclusions: The increased PGE2 and EP2/4 expression in spheroid MSCs plays a role in regulation of EP receptors and COX-2 expression, but the mechanism which leads to the initial upregulation of these genes is not fully explained by increased PGE2 signaling alone. This suggests, additional factors inherent in the spheroid structure, such as cell-cell contact or hypoxia, play integral roles in the development of the spheroid MSC phenotype.

459. Investigating Tumor-Homing Stem Cell Therapy for Post-Surgical Pediatric Brain Cancer

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Introduction: In face of the invasive and rapid-growing nature of Medulloblastoma (MB), the most prevalent primary pediatric brain tumor, genetically engineered Neural Stem Cells (NSCs) have emerged as a promising treatment. Although NSCs are known to migrate towards solid MB foci and delivery therapeutic agents to control tumor growth, investigations into the efficacy of NSC therapy against post-surgical MB, a central component of the clinical standard of care for the disease, remains poorly defined. To address this critical gap, we developed an image-guided mouse model of MB surgical resection. We then investigated multiple parameters of autologous and personalized NSC therapy for post-operative MB delivered either directly into the surgical resection cavity. **Methods:** First, Daoy and D283 human MB cells were transduced with viral vectors to express optical reporters. The cells were then implanted into the cerebellum of Nude mice, the subsequent established tumors were then resected using intraoperative image guidance. Following resection, NSCs derived from the mouse cerebellum (mNSC) and a novel induced NSCs (iNSCs) generated from pediatric skin were engineered to express cytotoxic agents, seeded into the post-operative cavity, and used to investigate intra-cavity therapy for post-surgical MB. **Results:** Real-time serial imaging showed tumor resection guided by fluorescence reduced MB volumes by 92%. In contrast to the gradual growth of pre-resection tumors, recurrent MB grew 3 times faster, re-developing 5 days after surgery. Exploring treatment of this aggressive post-surgical MB, iNSCs were capable of killing 92% of MB when tested *in vitro* co-culture assays. *In vivo*, seeding of cytotoxic mNSCs into the post-operative surgical cavity decreased MB volumes 15-fold and extended median survival 133%. Intra-cavity iNSC therapy suppressed post-surgical tumor growth and prolonged survival of MB-bearing mice by 123%. Lastly, in vitro real-time motion analysis assays showed that both iNSCs and mNSCs migrated specifically towards MB cells, with iNSCs migrating 10-fold faster and mNSCs 2-fold faster than the control counter parts. In vivo, the cells were found along the tumor tissue interphase or to be distributed throughout the core of the tumor when implanted in the contralateral hemisphere. **Conclusions:** Our study demonstrates that intra-cavity mNSC/iNSC therapy effectively targets residual tumor foci, suppressing re-growth of post-operative MB.

460. Therapeutic Genome Editing of Myotonic Dystrophy Type 1 Ips Cells for Personalized Cell-Based Therapy

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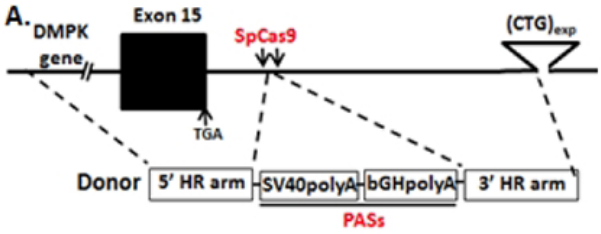
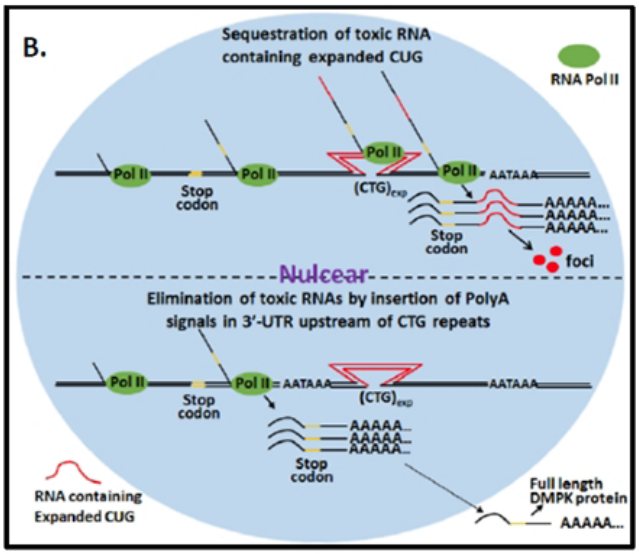
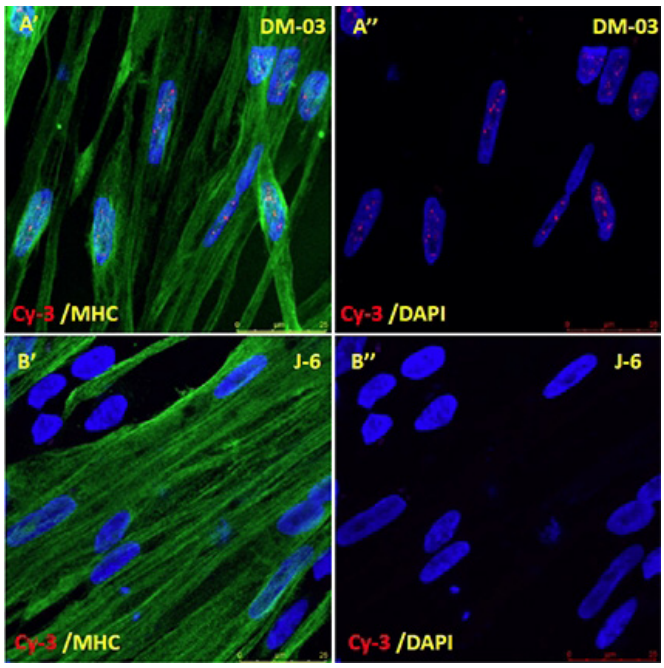
Introduction: With the advancement of genome-editing and induced pluripotent stem (iPS) cell technologies, there has been increasing enthusiasm for therapeutic application, particularly to monogenetic inherited disorders. Myotonic dystrophy (Dystrophia Myotonica, DM) type 1 (DM1) is caused by a CTG nucleotide repeat expansion within the 3'-untranslated region (3'-UTR) of the Dystrophia Myotonica protein kinase *(DMPK)* gene. The expanded CTG repeats encode toxic CUG RNAs that cause disease largely through RNA gain-of-function, which leads to aberrant gene splicing. For personalized cell-based therapy, it will be ideal to correct the mutation in iPS cells before subjecting them to multipotent or unipotent stem cells for cell transplantation. In this study, we explored therapeutic genome editing using CRISPR/Cas9 via targeted insertion of polyadenylation signals in the 3'-UTR upstream of the CTG repeats to eliminate toxic CUG RNAs in DM1 iPS cells ($$graphic 1). ***Methods:*** A CRISPR/SpCas9 nickase system was adopted to specifically insert polyadenylation signals in the 3'-UTR upstream of the CTG repeats. Genome-edited iPS cell clones were established using these strategies and the therapeutic effects of this genome manipulation on phenotype reversal were investigated in genome-edited DM1 iPS cells and their differentiated progenies. ***Results:*** The insertion of polyadenylation signals in the 3'-UTR upstream of the CTG repeats completely eliminated toxic CUG RNA repeats with normal processing of *DMPK* transcripts. This approach reversed the phenotypes in neural stem cells, neurons, cardiomyocytes and skeletal muscle myofibers derived from genome-corrected DM1 iPS cells ($$graphic 2). ***Conclusion:*** Targeted insertion

of polyadenylation signals in the 3'-UTR upstream of the CTG repeats is a viable approach for genome correction of DM1 iPS cells for personalized cell-based therapy.







461. Characterizing Patient-Derived Induced Neural Stem Cells as a Drug Delivery Platform for Glioblastoma

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Neural stem cells (NSCs) engineered to deliver drugs are a promising new approach to treating glioblastoma (GBM). In the clinic, the ideal NSC therapeutic should be easy to isolate and autologous to avoid immune rejection. Thus, we developed a method to directly transdifferentiate human fibroblasts into induced neural stem cells (iNSCs) using a single-factor Sox2 transduction strategy. The iNSCs are engineered using lentiviral vectors to express fluorescent reporters and the cytotoxic protein TRAIL. These cytotoxic iNSC-TRAIL attenuated tumor growth in surgically-resected human GBM xenografts *in vivo*, prolonging median survival from 22 to 49 days within 3 weeks post-infusion. However, iNSC generation has been characterized primarily using a normal human fibroblast cell line. The generation of iNSCs and subsequent inter-patient variability in transduction efficiency, conversion efficiency, migratory capacity, and therapeutic efficacy of this approach to GBM treatment remains unexplored. The goal of this study was to generate and characterize GBM patient cells to optimize our rapid transdifferentiation protocol and dosing regimen in a patient-specific manner. The isolation process began by collecting small skin punch biopsies from the surgical border of human GBM patients undergoing tumor debulking at UNC Hospitals. We then processed the tissue to isolate and bank fibroblast lines from 6 different patients. We first discovered using cell viability assays that the different lines grew at different rates, with the slowest increasing only 1.27-fold and the fastest 2.98-fold over 6 days. Viral transduction is a central component of iNSC generation, and using the same viral titer of a fluorescent reporter on each patient line resulted in marked differences in the percentage of positive cells, ranging from only 22.45% positive to 87.22% positive depending on the patient sample. Immunofluorescent staining also showed differences in Sox2 expression 5 days post-transdifferentiation. Lastly, we explored the efficacy of GBM patient-derived iNSC therapy *in vivo*. Human patient-derived GBM cells were implanted in nude mice. 3 days later, the established tumors were surgically resected and GBM-patient iNSC were delivered into the surgical cavity on scaffolds. Using the patient sample with the highest transduction efficiency and growth rate, we found that mice treated with control iNSCs increased 615-fold 3 weeks post-resection. In contrast, cytotoxic iNSC therapy reduced GBM growth to only 16-fold. Additionally, iNSC therapy extended median survival from 28 to 40 days post-resection. Together, these results provide the first evidence that iNSCs can be generated from cancer patient tissue, reveal differences between patient lines, and demonstrate the efficacy of this approach in mouse models of surgical resection. This suggests that generation of therapeutic iNSCs from patient-derived tissue has the potential to be an efficacious approach to treating cancer.

462. B Cell Based Cell Therapy: Clinical Grade Production of Human B Cells for the Treatment of MPS I

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Sustained, *in vivo* production of therapeutic proteins is an effective approach for the treatment of numerous diseases. While viral based methods have most commonly been employed, they are expensive and are not expected to allow for repeated dosing. To overcome these limitations, we have developed a clinic ready production platform that incorporates genetic modification of B cells using the *Sleeping Beauty* (SB) transposon system. Our first application of this technology is for treatment of Mucopolysaccharidosis Type I (MPS I), caused by deficiency of the lysosomal hydrolase alpha-L-iduronidase (IDUA), resulting in systemic accumulation of glycosaminoglycans. *In vivo* efficacy experiments in NSG MPS I mice have resulted in mean IDUA plasma levels up to 15 times the wild type level (about 1 nmol/hr/ml) and human IgG plasma levels ranging from 200 μg/mL up to 1 mg/mL, providing evidence of B cell adoptive transfer. Multiple peripheral tissues harvested 60 days post infusion have shown two- to ten-times the normal level of IDUA with normalization of glycosaminoglycan storage, demonstrating significant metabolic correction. In support of clinical translation of SB-engineered human B cells for the treatment of MPS I, we have completed several GMP grade human scale engineering runs and GLP toxicology studies are currently underway. Collectively, our efforts highlight the utility of the B cell platform for the treatment of MPS I and the potential for application in a wide array of diseases.

463. Robotic High Throughput Production of Patient Derived iPSCs for Autologous Photoreceptor Cell Replacement

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Disorders such as age-related macular degeneration (AMD), retinitis pigmentosa (RP), Stargardt Disease and Leber Congenital Amaurosis are amongst the most common and debilitating forms of vision loss in the western world. As these diseases are characterized by death of the light sensing photoreceptor cells of the outer neural retina, to restore useful vision some form of photoreceptor cell replacement will likely be required. With each of these disorders having documented abnormal activation of the immune system, immunologically matched patient derived induced pluripotent stem cells (iPSCs) would be the ideal cell source for such an application. Unfortunately, one of the greatest limitations of autologous iPSC based cell replacement is that patient specific therapeutics are difficult to produce in large numbers using traditional manufacturing strategies that are designed for large batch production of a single product for the treatment of an entire patient population. As such, new manufacturing strategies and standard operating procedures specifically designed for high throughput generation, genetic correction and differentiation of patient-specific iPSCs is required. In this study we describe the use of custom designed robotics and ISO Class 5 cGMP cell culture atmospheric isolators for the high throughput production of clinical grade patient specific iPSCs. Briefly, patient derived fibroblast cell lines were generated from 27 individuals with inherited retinal degenerative blindness, ranging from 6 to 92 years of age (i.e., 3 patients from each decade of life). All steps subsequent to generation of fibroblasts were performed using a custom robotic cell culture platform called the CellX. For iPSC generation 200,000 fibroblast were plated into 1 well of a 6 well tissue culture treated cell culture dish. At 24 hours post-plating cells were transduced with Sendai virus (Cytotune 2.0 at an MOI of 5) designed to drive expression of the transgene OCT4, SOX2, KLF4 and c-MYC. At 18-24 hours post-transduction cells were washed and fed with fibroblast cell culture media. At 48 hours post-transduction cultures were fed with equal parts fibroblast cell culture media and complete E8 media. Cultures are subsequently fed daily with E8 media and passaged at day 5 onto laminin 521 coated 6 well culture plates at a density of 35,000-65,000 cells per well in E8 media. Over the next 21 days cultures were fed daily and imaged every 3-5 days using the CellX robot. Over this time period transduced fibroblasts underwent the stereotypical morphological changes resulting in the formation of iPSC colonies large enough for robotic isolate and clonal expansion. Colonies were picked from the donor plate and transferred into a 12 well laminin 521 coated recipient plate. From there clones were expanded in 6 well laminin 521 coated cell culture dishes. Pluripotency was confirmed at passage 10 via scorecard analysis and embryoid body formation. Genetic integrity was confirmed via karyotyping. These results demonstrate that we have successfully developed a high throughput robot iPSC generation system and accompanying standard operating procedures that are compatible with current good manufacturing practices. This system will enable clinical grade production of iPSCs for autologous retinal cell replacement.

464. Alterations in PGE2 and IL-6 Production Partially Drive the Pro-Inflammatory Effects of Palmitate Exposed Mesenchymal Stromal Cells

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Type 2 diabetes (T2D) has risen at an alarming rate in the US, with 1 in 3 Americans now diagnosed as diabetic or pre-diabetic. Though T2D can itself lead to a complex range of adverse health outcomes, what has become increasingly evident is the modifying effect of T2D on comorbid disease course and therapeutic efficacy. Our recent work demonstrates that palmitate, a specific component enriched in the serum of T2D patients, has a powerful and diverse effect on mesenchymal stromal cells (MSCs), including increasing transcription of pro-inflammatory mediators like PTGS2 and IL6. Importantly, we discovered that palmitate not only dampens MSC immunosuppressive

efficacy, but converts MSCs to a pro-inflammatory phenotype, which can be prevented by pre-licensing MSCs with IFNg and TNFa. In the present study, we aimed to determine how specific phenotypic changes in palmitate-exposed MSCs contributed to the permissive, pro-inflammatory effect observed in our previous studies.

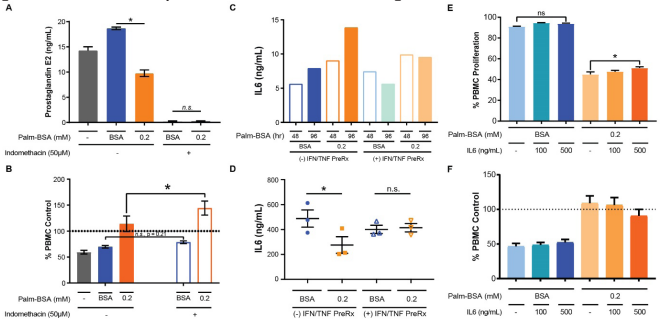

Figure 1. Palmitate Alters the MSC Secretome, with Significant Changes Occuring in MSCs Alone and in Co-culture with PBMCs.

To determine the role of PGE2 in suppression assays involving PBMCs, supernatants from MSC-PBMC co-cultures were analyzed by PGE2 ELISA with or without indomethacin, a PTGS2 inhibitor, and T cell proliferation was quantified. We found that PGE2 levels in co-cultures were significantly impaired by palmitate exposure, leading to a 49% drop in PGE2 (Fig. 1A). Additionally, we found that blocking the production of PGE2 through indomethacin treatment led to an exacerbation of the pro-inflammatory effect of palmitate exposure, causing a 30% increase in T cell proliferation over palmitate-exposed non-indomethacin treated control (Fig. 1B). This data implicates dysfunction of PGE2 production as a contributing factor in the pro-inflammatory effect observed in palmitate exposed MSC-PBMC co-cultures. IL6, another MSC secreted factor, was also elevated transcriptionally after palmitate exposure. To understand if pre-licensing changed the production of IL6 in MSCs alone, pl-MSCs and naïve MSCs were exposed to palmitate followed by ELISA quantification of IL6. IL6 ELISA was also performed on the supernatant of MSC-PBMC co-cultures with pl-MSC and naïve MSCs. Lastly, varying doses of IL6 were added to MSC-PBMC co-cultures to determine if the addition of IL6 alone could drive the pro-inflammatory effect previously observed. Palmitate exposure in naïve MSCs led to a large increase in IL6 (Fig. 1C), which was consistent with the transcriptional increase we had previously seen. Interestingly, we found that pre-licensing prevented this palmitate-induced increase in IL-6; however, high levels of IL6 were still produced. In contrast, in MSC-PBMC co-cultures with naïve MSCs, palmitate exposure led to a decrease in IL6 production, while pl-MSCs did not alter IL-6 levels in response to palmitate (Fig. 1D). With IL6 addition, PBMCs cultured alone showed increased proliferation (Fig. 1E); however, in MSC-PBMC co-culture, no significant differences were observed in PBMC proliferation, though there was a modest trend toward high levels of IL-6 suppressing PBMC proliferation in co-culture (Fig. 1F). In sum, these data highlight an altered, dysfunctional MSC secretome in the presence of palmitate, which is further exacerbated by crosstalk between MSCs and PBMCs.

465. AAV Mediated High-Efficiency Genetic Engineering in Stem Cell Organoids

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Introduction: Three-dimensional (3D) organoids from tissue-specific progenitors, induced pluripotent stem cells, or embryonic stem cells have been considered as potential tools to explore the roles of stem cells in tissue genesis. In addition, organoid systems could be used to reconstruct disease development and to develop novel therapeutics. Most importantly, 3D organoids have great potential in clinical drug screening for the purpose of precision medicine. Unfortunately, the wide use of these systems is limited largely due to the inefficiency of genetic manipulation. **Methods and Results:** To overcome the current drawbacks, we used intestinal stem cell organoids and bile duct stem cell organoids. First, various recombinant adeno-associated virus (rAAV) serotype vectors containing either GFP or mCherry report genes were compared and the transgene expression was obtained using confocal microscopy. It was evident that rAAVDJ was the most significant one to high-efficiently transduce both organoids. The transduction efficiency and intracellular vector genome numbers were both in a dose-dependent manner determined by flow cytometry and quantitative PCR assays, respectively. Point mutation of surface tyrosine and serine residues further enhanced the viral-mediated transgene expression and led to more than 95% transduction at an MOI of 10,000 vgs/cell. The cell entry and intracellular trafficking of viral particles were then investigated by small molecules against lipid-raft, dynamin-dependent and CLIC/GEEC-associated endocytosis, endosome acidification, and Golgi transport. Moreover, both organoids showed more than 95% transduction efficiency at Day 3 post-viral infection, whilst only ~3% of GFP positive cells were remaining at Day 14 by flow cytometry analysis, compared to ~2% of mock treated cells. The gradual loss of viral genome numbers was further confirmed by quantitative PCR assays. The above data suggested that rAAVDJ vectors are extremely useful for exploring the tissue genesis of stem cell organoids. To this end, HNF4α and Alk5 was individually over-expressed in the bile duct stem cell organoids using rAAVDJ vectors. Our results clearly demonstrated that Alk5 is involved in the differentiation of bile duct stem cell to hepatocytes, presumably due to the activation of TGFβ signaling pathway. **Conclusion:** Taken together, we have developed a novel gene manipulating approach for intestinal and bile ductal organoids, which allows high-efficient gene overexpression, knock-down and genetic editing *ex vivo*. The combination of organoid culture with rAAV vector will be a broadly applicable tool to study tissue homeostasis and disease, complementing classical conditional mouse models. As organoid transplantation has been employed to repair endodermal epithelia injury *in vivo*, our system also shows great therapeutic potential in regeneration medicine.

466. Cell Sheets from Adipose-Derived Mesenchymal Stromal Accelerate Healing and Angiogenesis

Pavel Makarevich[1,2], Natalia Alexandrushkina[1], Konstantin Dergilev[2,3], Evgeny Shevchenko[2,3], Maria Boldyreva[2,3], Peter Nimiritsky[1], Olga Grigorieva[1], Anastasia Efimenko[1,2], Yu-Chen Hu[4], Yelena Parfyonova[2,5], Vsevolod Tkachuk[1,2]

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Cell sheet (CS) technology has advantages for application in regenerative medicine as far as it allows effective and feasible delivery cells for therapeutic application.Cell sheets are minimal tissue-engineered constructs that consist of viable cells and extracellular matrix proteins forming a solid multilayered structure. Their expressed therapeutic potential relies on better survival after delivery compared to injection of dispersed cells. This approach has been in our field of interest during last years and we have developed CS application from adult stem cells - mesenchymal (MSC) and c-kit+ cardiac stem cells (CSC) for stimulation of angiogenesis in ischemic skeletal muscle and infarcted myocardium, induction of wound healing and other clinically relevant models. Starting from wound healing in a rat model of deep defect of soft tissues we found that delivery of constructs from adipose-derived MSC significantly accelerated wound healing compared to suspended cells accompanied by higher engraftment rate of CS-delivered MSC compared to dispersed.We also successfully adopted the concept of gene delivery application to modify cells for sake of new therapeutic methods. Using viral delivery of growth factors allows to enhance MSC paracrine and regenerative potential for increased efficacy and better safety. Mostly, these methods utilize the crucial role of paracrine stimuli generated by stem cells during tissue repair or regeneration. Over last decade we have been elaborating to develop strategies using viral vectors to increase production of growth factors and "tune-up" the cells pro-regenerative capacity. Using adeno-associated viruses (AAV) and baculovirus we managed express growth factors in MSC and CSC. Developed methods of viral delivery to express VEGF165 allowed to increase pro-angiogenic potential of cells and induce effective angiogenesis in ischemic tissue of experimental animals rendering effect that was significantly higher compared to GFP-treated or un-modified cells. Indeed, using nude mice to evaluate human MSC impact on recovery of blood flow, we found that VEGF-expressing MSCs had better survival and resulted in higher perfusion and blood vessel counts at experiment's endpoint.However, viral modification of constructs resulted in even better functional outcome in animal models of ischemia compared to untreated cells or injected dispersed. Furthermore, a large subset of data was obtained indicating graft/host interactions, vascularization of implanted construct and limited cell proliferation within the tissue layer.Overall our set of studies summarizes efforts to develop a next-level cell therapeutic combining feasible and effective delivery technique with viral modification as a way to enhance paracrine modality known to be crucial for MSC and their regenerative potential in numerous lesions affecting human population.

Study was supported by RFBR grant #17-04-01452 (cell culture and in vitro assays), partially- by RSF grant #16-45-03007 (animal test and histology) and used biomaterial biomaterial collected and preserved in the frame of the project "Scientific basis for national bank-depositary of living systems" (RSF agreement #14-50-00029) using the equipment purchased as a part of Lomonosov Moscow State University Program of Development.

467. Improving Clinical Translatability by Selecting MSCs with an Immunomodulatory, Secretory Signature

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Mesenchymal stromal/stem cells (MSCs) have demonstrated potent immunomodulatory, anti-inflammatory, and trophic effects making them ideal candidates for comprehensive treatment of numerous clinical indications. MSCs have the innate ability to sense and respond to their environment and, in turn, mediate as a dynamic signaling source. Subsequently, cells surrounding damaged tissues repair and restore homeostasis to the local milieu, and immune tolerance is promoted by immunomodulation in lymphoid organs. For rapid enhancement of MSCs effects, we predisposed MSCs to inflammatory mediators *in vitro*, a method considered as "priming", resulting in characteristic phenotype with robust anti-inflammatory, immunomodulatory properties. Furthermore, we identified an intrinsic subpopulation of MSCs with a "signature phenotype" that demonstrated comparable effects to those seen with primed MSCs. By magnetic-activated cell sorting of MSCs using CD146, we performed fractionation of MSCs subpopulations. Following, naïve, primed, and fractionated MSC subpopulations were cultured with human T cells or macrophages, and stimulation assays were performed. Primed MSCs and $CD146^+$ fraction exhibited a highly anti-inflammatory secretory profile with a marked ability to suppress T cell-mediated activities, including proliferation, differentiation, and function. Moreover, these MSCs were capable of skewing the M1/M2 axis of activated pro-inflammatory macrophages (M1) to the immunosuppressive phenotype (M2). We further defined a consistent, reproducible, and distinguished cellular phenotype of primed MSCs ($CD146^+$; $CD107^+$; $CXCR4^+$; LIF^+) that has a robust secretory capacity, an enhanced immunomodulatory capacity, and release exosomes enriched with regulatory miRNAs. Together, this evidence suggests a priming method that produces a highly effective, reproducible, uniform, and therapeutic phenotype of MSCs with superior immunomodulatory properties. Our study provides translational, evidence-based research using MSCs as promising candidates for the comprehensive treatment of immune system- and inflammation-mediated pathologies.

468. Impact of Different Culture Media Supplements on CAR T Cell Function

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Although the clinical failure/success of CAR T cell therapy is not always well understood, the field continues to learn what biological features are associated with a clinical response. Today a biological attribute accepted by different groups as a feature that correlates with long-term T cell persistence and clinical efficacy, is the presence of a naïve (T_N) and central memory (T_{CM}) T cell phenotype. In this study we explored the impact of different media supplements on the phenotype of CAR T cells. We used, as a platform, second generation CAR targeting prostate stem cell antigen (PSCA) with CD28/CD3z endodomain, which has been developed in our lab. PBMCs were stimulated with plate-coated OKT3/CD28 antibody and expanded in the presence of IL2 with 10% fetal bovine serum (FBS), then transduced with CAR-PSCA. After 3 days of transduction, CAR T cells were split into growth conditions with different supplements: FBS, AB serum (ABS) and pathogen reduced human platelet lysate (PR HPL) at concentrations ranging from 2.5% to 10%. We evaluated CAR T cell growth, phenotype and short- and long-term *in vitro* killing ability. We monitored T cell expansion over a 21-day period and found that CAR T cell growth was similar among the conditions except 2.5% and 5% FBS, in which we observed much less T cell expansion. In addition, in a short-term *in vitro* killing assay (5 hrs ^{51}Cr release assay) CAR T cells cultured in the different conditions exhibited comparable cytolytic ability. Interestingly, populations of CCR7+ cells (T_N and T_{CM}) were dramatically increased when CAR T cells were maintained in 10% PR HPL (CD4+: 62.8±3.2%; CD8+: 74.0±4.0%) compared to 10% FBS (CD4+: 31.4±2.7%; CD8+: 17.5±1.2%) and 10% ABS (CD4+: 15.7±2.8%; CD8+: 7.9±1.5%). Consistent with the greater presence of CCR7+ cells, in a long-term (9 days) coculture killing assay, CAR T cells maintained in PR HPL showed a potent anti-tumor effect with greater expansion of T cells (Tumor cell fold-expansion, 10% PR HPL: 1.6±1.3, 10% FBS: 7.1±3.5, 10% ABS: 5.4±3.4; T cell fold- expansion, 10% PR HPL: 9.4±1.5, 10% FBS: 3.3±0.5, 10% ABS: 7.5±2.0). Our work suggests that culturing CAR T cells with human platelet lysate may lead to improved maintenance of undifferentiated T cells with enhanced persistence and in-vitro anti-tumor killing ability.

469. A Novel Non-Integrating Non-Viral DNA Vector for the Persistent Genetic Modification of Embryonic and Hematopoietic Stem Cells

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Introduction: The capability of stem cells to differentiate into all types of specialized progeny, hold great promise for the future of gene therapy and regenerative medicine. The most efficient way to genetically modify a cell population is by targeting the originating population or embryonic stem cells, which can propagate infinitely and can pass on genetic modifications to their progeny. One example is the targeting of hematopoietic stem cells (HSC) for the genetic correction of hematopoietic diseases. A typical way to genetically modify pluripotent cells is by infecting stem cells with integrative vectors. Viral vectors have been the tool of choice to modify pluripotent cells, however a major drawback to their use is their potential integrative mutagenesis and that they typically become silenced either directly at the stem cell stage or during differentiation. Here we demonstrate that a novel non-viral and non-integrating technology based on a Scaffold Matrix Attachment Region (S/MAR), can successfully genetically modify embryonic and hematopoietic stem cells and that transgene expression is persistently sustained through differentiation.**Results:** Murine embryonic stem cells (mESC) were stably labeled with an episomal GFP-S/MAR encoding vector, which provided robust and sustained levels of transgene expression. We outruled possible influence of the vector in the stem cell capabilities by analyzing a panel of pluripotency markers. We then performed a directed *in vitro* differentiation from ESC to hematopoietic precursors, in which we observed sustained expression of our DNA vectors throughout the process without observing transgene silencing . We further challenged the capabilities of the DNA Vector labeled stem cells by generating chimeric animals. The transgenic organs, including hematopoietic tissues, showed high levels of transgene expression. Our data demonstrates that S/MAR DNA vectors can sustain episomal transgene expression from embryonic stem cells to fully differentiated hematopoietic tissues without silencing, vector loss or integration. **Conclusions:** For the first time, we have shown that a non-viral episomal vector based on mammalian chromosomal elements is capable of genetically modifying embryonic stem cells whilst avoiding vector loss or differentiation-mediated transgene silencing. We demonstrate that this DNA vector system provides robust and sustained transgene expression in pluripotent cells during hematopoietic differentiation, without damaging or altering the stem cells' properties. Our vector system represents an alternative tool for 1) the modification of HSC and the treatment of hematopoietic diseases; 2) for gene therapy approaches that rely on robust and continuous expression of transgenes; as well as 3) a cell source for replenishment of HSC in diseases in which they are affected or absent.

470. CD200/CD200r Enhances the Capacity of Plancetal Mesenchymal Stem Cells to Protect Vascular Endothelial Cells from a Lipopolysaccharide-Induced Cell Injury

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Abstract: Acute Lung injury (ALI) remains a devastating syndrome caused by overwhelming inflammatory responses with a mortality rate over 50% in China. Recent studies demonstrated that mesenchymal stem cells (MSCs) exerted their biological functions of immunosuppression injury repair, mainly through the realease of extracellular vesicles (MSC-EVs) that containing factors and genetic contents with various biological activities, including mRNAs and microRNAs (miRNAs). CD200 is a highly conservative type I transmembrane glycoprotein, which functions as immunosuppressive molecules through binding to CD200R immunosuppressive signals. The CD200/CD200R signaling is able to regulate inflammatory response threshold and maintain the immune and tissue homeostasis. Our previous studies revealed that more abundant CD200 protein was expressed in human fetal placenta mesenchymal stem cells (fPMSCs) in comparison with PMSCs of mother origin (mPMSCs). Importantly, fPMSCs exhibited significantly stronger capacity of immune regulation compared to mPMSCs, indicating that the CD200/CD200R signaling played key roles in immune regulation of MSCs. In order to uncover whether the CD200/CD200R signaling play a major role of fPMSCS in the protection of cells from injury, the effect of CD200 of fPMSCs was investigated in a lipopolysaccharide (LPS)-induced vascular endothelial cell (VEC)/fPMSC co-culture injury model. By using a lentiviral vector-mediated gene transfer approach, fPMSCs overexpressing CD200 (CD200-positive fPMSCs) and CD200 gene silenced fPMSCs (CD200-negative fPMSCs) were generated. The results showed that CD200-positive fPMSCs could significantly increased the cell vitality of VECs exposed to LPS, in comparison with the control; in contrast, CD200-negative fPMSCs dramatically reduced the vitality and proliferation of LPS-treated VECs compared to the controls ($p<0.01$). Hoechst staining and cytometric analysis further demonstrated that strikingly reduced and increased the LPS-induced VEC apoptosis and production of cytokine IL-8 and TNF-α were observed in co-culture models of VEC/ CD200-positive fPMSCs and /CD200-negative fPMSCs as compared with controls, respectively. Mechanistically, the co-culture of CD200-positive and -negative fPMSCs led an alleviated and enhanced LPS-induced Toll-like receptor signaling (TLR) activity, along with a reduced and increased expression of NF-κB and apoptotic proteins in VECs, respectively. These studies will provide an evidence of the importance of CD200/CD200R activity in immunosuppressive property of MSCs, suggesting that the fPMSCs may be a promising MSC cell type with greater potential for treatment of ALI. This work was supported by a grant from the National Natural Science Foundation of China to JW (No. 81460247).

471. Procell™ Microbiome Treatment of Bone Marrow Mononuclear Cells Endows Neuroprotective Activity in EAE Model of Multiple Sclerosis

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Introduction: Immune modulation by bone marrow derived mesenchymal stem cells (MSC) has been shown to reduce progression in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis. ProCell, a peptide derived from healthy microbiome was previously demonstrated to augment regenerative activity of stem cells. The current study sought to determine whether ProCell treatment of syngeneic bone marrow cells possesses ability to reduce progression of EAE. **Methods**: Immune modulatory activity of ProCell was assessed by treatment of bone marrow mononuclear cells with ProCell and subsequently assayed for proliferative ability and cytokine in response to CD3/CD28 stimulation. The concentration and incubation time of ProCell identified for maximal immune modulatory effect was used to treat syngeneic bone marrow cells prior to implantation in the SJL/J model of EAE. Cell transplant experiments involving transfer of mesenchymal stem cells (CD105), monocytes (CD14), T cells (CD3) and B cells (CD20) where performed to identify the cellular population responsible for disease protective effect. **Results:** ProCell induced an inhibitory effect on CD3/CD28 stimulated T cell proliferation which was associated with upregulation of IL-10 and TGF-beta and inhibition of IFN-gamma and IL-12 production. Dose dependent inhibition of EAE and accelerated reduction of relapse was observed in animals treated with ProCell cultured cells. Correlation with disease inhibition was associated with increased FoxP3 expressing cells. Adoptive transfer experiments revealed disease inhibition was associated with monocytic cells. **Conclusions:** These data suggest that culture of bone marrow mononuclear cells in ProCell results in expansion of an monocytic population capable of immune modulation. Elucidation of whether immune modulation is mediated in an antigen specific or non-specific manner is under investigation.

472. DOR Rescues Human MSCs Subjected to 'Serum Free' Apoptotic Condition and Enhances Anti-Inflammatory Effect in Part via the DOR/PI3K/Akt Pathway

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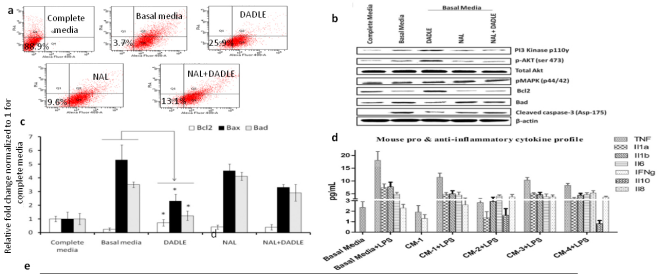
Aim: Nutritional deprivation and inflammation-rich zones are the major causative reasons for poor survivability of transplanted mesenchymal stem cells (MSCs). Therefore, in the present study, we demonstrated the cytoprotective and anti-inflammatory effects of activated delta (δ)-opioid receptor (DOR) with synthetic peptide [DAla2, D-Leu5]-enkephalin (DADLE) treatment on human MSCs cultured in serum-starved condition. **Main methods:** Cell viability was measured using MTT and Annexin V/PI assays. Expressions of pro-

apoptotic (Bcl2) and anti-apoptotic genes (Bax/Bad), levels of activated p44/42 MAPK, Akt, PI3-kinase-p110γ and cleaved caspase-3 were determined by qPCR and western blot. Levels of secreted cytokines were measured by ELISA. **Key findings:** In comparison to the control, DADLE significantly increased cell survivability under serum deprived condition as confirmed by MTT (71% vs 45%) and Annexin V/PI assays (25.9% vs 3.7%). Significant upregulation of pro-apoptotic Bcl2 (~2.1 folds), down-regulations of anti-apoptotic Bax/Bad (~2.6/2.7 folds) as well as of cleaved caspase-3, increased expression of PI3kinase subunit p110γ and activation of Akt (Ser473) were observed following DADLE treatment in cells under 'serum deprivation' stress. In addition, DADLE treated hMSCs secreted increased levels of anti-inflammatory cytokines (IL10/IL4/TGF-β) under serum deprived condition. LPS stimulated macrophages showed abated release of pro-inflammatory cytokines (IL1/TNFα/IL6) when grown in hMSC conditioned 'serum deprived' media treated with DADLE. Both the cytoprotective and anti-inflammatory effects of DADLE were inhibited by the DOR specific antagonist naltrindole. **Significance:** The DOR signaling pathway improved cell viability and enhanced anti-inflammatory effect of hMSCs subjected to 'serum deprivation' stress that could have potential therapeutic benefits in reparative medicine.



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Groups	Pro-inflammatory cytokines (pg/mL)		Anti-inflammatory cytokines (pg/mL)		
	TNF	*Il6*	*Il4*	*Il10*	*TGF-β*
CM-1: basal media only	1.13 (± 0.23)	2.4(± 0.15)	0	1.32(± 0.24)	3.1(± 0.51)
CM-2: basal Media + DADLE	0	0	3.73(± 1.13)	6.54(± 1.12)	12.1(± 2.71)
CM-3: basal media + Nal	1.23(± 0.13)	1.71(± 0.65)	0	2.09(± 0.31)	3.9(+/− 1.5)
CM-4: basal media + DADLE + Nal	1.34(± 0.21)	0	1.69(± 0.39)	3.1(± 1.06)	7.16(± 2.08)

Figure: 1 a. Flow cytometric analysis of Annexin-V/PI stained cells grown in the different treatment conditions (5×10^4 cells/well was plated in 6 well plates). All 4 groups were cultured for 48h followed by apoptosis evaluation. *$p < 0.05$; **b.** Representative western blot images of PI3K-110γ, pAkt (ser 473), total Akt, Bcl2, Bad, phospho p44/p42-MAPK (Thr202/Tyr204) and β-actin done from HMSCs grown under the different treatment conditions; **c.** Quantitative-PCR analysis of anti-apoptotic gene Bcl-2 and pro-apoptotic genes Bax and Bad in hMSCs grown under the different treatment; **d.** Pro and anti-inflammatory cytokines secreted by hMSCs under the different treatment conditions; **e.** Expression profiles of mouse pro and anti-inflammatory cytokines. RAW-264.7 cells were cultured in the different conditioned media (CM) with or without LPS for 3 h. The supernatants were then collected and analyzed for the different cytokines (ELISA).

473. Solutions for Cell Therapy Production

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The long-term view of regenerative medicine therapies predicts an increased need for expansion solutions that ease scalability, utilize animal origin-free materials and are compatible with limited downstream processing steps. As more cell therapeutics progress through clinical testing, current *in vitro* culture methods are proving cumbersome to scale and lack robustness. Moreover, high quality animal origin-free reagents and downstream processing support the future implementation of production solutions that will be required following clinical success. Here, we describe the implementation of single use bioreactors and high-quality reagents for expansion of cell therapies. We include examples from allogeneic mesenchymal stem cells (hMSCs) and induced pluripotent stem cells (iPSCs). The bioreactors supported expansion of both cell types: hMSCs were cultured on microcarriers whereas iPSCs were cultured as aggregates circumventing the need for microcarriers or other substrates. The presentation reviews solutions for expansion of these different cells within the context of various upstream process development steps as well as scaling and processing with high yield, recovery, viability and expected cell characteristics. The presented examples demonstrate how start-to-finish solutions for manufacturing, including high quality reagents, are key enabling technologies for success in industrializing cell therapies.

474. Human Umbilical Cord as a Source of Multiple Potential Therapeutics to Treat Experimental Traumatic Brain Injury

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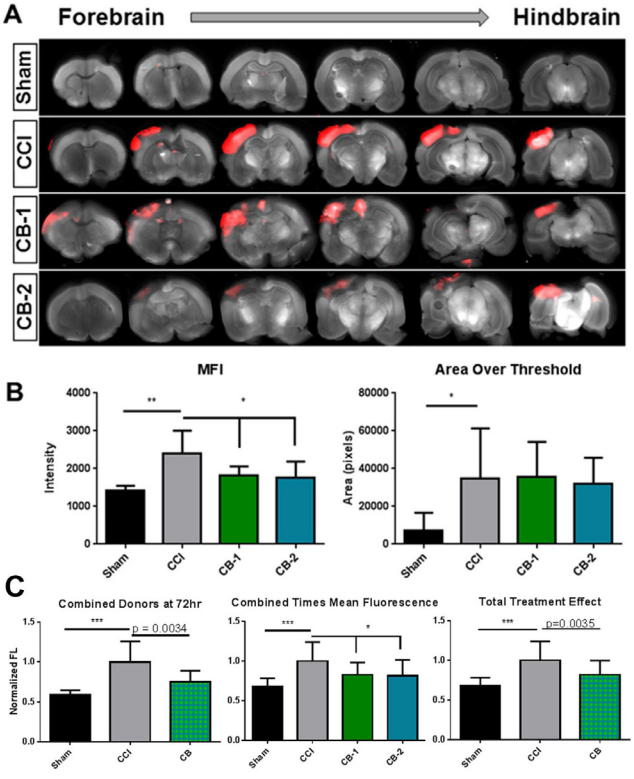
Traumatic brain injury (TBI) is a wide reaching condition that affects millions of athletes, soldiers, and civilians daily resulting in an incredible expense including clinical expense, rehabilitation, lost productivity, decreased quality of life, and long-term complications. Treating TBI effectively has been elusive and difficult. While there have been many clinical trials, and extensive pre-clinical studies, there are still no FDA-approved therapies for TBI. There is a growing public interest in using cord blood-related therapeutics due to the increasing availability of cord blood in public and private banks. Here we describe a series of studies investigating the potential of privately banked and cryopreserved human umbilical cord-derived products, or extracellular vesicles isolated from those products, to treat TBI in a controlled cortical injury rat model. Human umbilical cord blood cells (hUCB), umbilical cord blood-derived extracellular vesicles (hUCBEv), umbilical cord-derived mesenchymal stromal

cells (ucMSC), and ucMSC-derived extracellular vesicles (ucMSCEv) were evaluated for their immunomodulatory activity in series of primary activated leukocyte assays, briefly profiled for potential mechanisms of action, and then used to treat TBI. The resulting effects on sub-acute blood-brain barrier permeability and neuroinflammation were then quantified as early biomarkers of outcome. This study highlights the versatility of banked umbilical cord blood and tissue as a source for potential autologous and allogeneic therapeutic products for TBI and possibly additional CNS injuries and conditions. Figure Legend: **hUCB to treat TBI.** Human umbilical cord blood cells were infused into the tail vein 72 hrs after a unilateral controlled cortical impact injury (CCI). **A.** Blood-brain barrier permeability is visualized using a fluorescent dye extravasation assay 96 hrs after injury. **B.** Dye extravasation of two different donors was quantified using both the mean fluorescence intensity (MFI) and by measuring the area of dye extravasation over the background threshold. **C.** The effects of two different cord blood donors were combined for a treatment effect (left), treatment at 24 hr and 72 hr was combined (center), and the total treatment effect from both cord donors and both treatment times were pooled (right).



475. Best Practices in the Development of cGMP Compliant Manufacturing Processes for Autologous Immunotherapies

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Autologous Cell and Gene Therapy (CGT) processes present unique challenges that require special design considerations across the entire manufacturing process and Chemistry, Manufacturing and Controls (CMC) strategy from tissue receiving and handling to final formulation, release testing and logistics. A consistent and cGMP compliant manufacturing process is the foundation for production of high quality cell therapy products. In order to ensure consistent safety and quality of any cGMP product, it is necessary to identify and assess key process parameters (KPPs) and map the impact of all expected KPPs on Critical Quality Attributes (CQAs) during process development to define the process design space. Only after the deconstruction of the effect of process on product can a robust, reproducible, and cGMP compliant process be developed. Here we highlight some of the key design considerations that can be implemented in the development of cGMP compliant manufacturing processes for a variety of autologous applications. By implementing appropriate process optimization and process control strategies along with incorporating innovative technologies during the development phase, we are able to address some of the key challenges in the current manufacturing processes including the use of open, manual, and inefficient small-scale processes. The evaluation of process yield, evaluation of the population of target cells and impurities, ability to scale out, and facility footprint are important parameters that are routinely studied during process optimization. In particular, understanding the biological variability of the cell source material is key to determining the manufacturing process tolerance to produce a CGT product within the defined release specifications. The impact of donor-to-donor variability on the KPPs must be investigated adequately during process optimization activities, and controlled in the process through implementation of a process control strategy. Most importantly, a well-designed testing strategy is required to implement any process control strategy and to ensure adequate characterization of the final product and must be achieved by developing and implementing reliable analytical methods for in-process monitoring and release testing. Autologous cell therapies also demand rapid release of the product, which presents further challenges. The optimization, qualification, and validation of the test methods are key steps that need to be completed depending on the release strategy and phase of application for CGT products.

476. Comparative Study of Xeno and Xeno-Free Culture Condition on Human MSCs and iPSCs

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Xenogenic materials, such as mouse embryonic fibroblast(MEF)s, FBS, porcine trypsin, used in stem cell culture cause transmission of microbial contaminants as well as immune rejection by high contents of xenogenic proteins. To minimize these safety issues, many xeno-free media have been developed. In the present study, we established xeno-free culture method using BM-MSCs and iPSCs and analyzed characteristics of stem cells with different culture conditions. In case of MSCs, we used bone marrow-derived MSCs(BM-MSCs). After establishing xeno-free culture condition, we analyzed genetic stability using karyotyping analyses. BM-MSCs showed normal karyotyping. Next, we performed cell proliferation ability test. The proliferation ability of BM-MSCs in xeno-free condition was better than FBS-based culture condition. In case of iPSC, we established xeno-free culture condition with no MEFs. We carried out AP staining and immunocytochemistry. iPSCs showed normal karyotyping. To elucidate the differentiation potential of iPSCs, we analyzed expression level of differentiation marker using the RT-PCR and immunostaining. We did not observed any difference with mRNA and proteins expression of ectoderm, mesoderm, endoderm markers. Further study is required for establishing a evaluation criterion of different culture condition.

477. Procell™ Microbiome Modified Bone Marrow Cells Possess Augmented Angiogenic Activity

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Introduction: The microbiome has been demonstrated to play a fundamental function in control of numerous biological activities. The current study sought to evaluate whether administration of microbiome derived peptides would modulate stem cell activity. **Methods**: Peptides and small molecules associated with healthy microbiota where assessed for ability to stimulate human umbilical vein endothelial cell (HUVEC) proliferation in vitro. Selected compounds where tested for ability to stimulate colony and tube formation. In vivo angiogenic activity was tested by administration of bone marrow mononuclear cells alone, or ProCell-treated bone marrow mononuclear cells in the hindlimb ischemia assay. **Results:** Of 73 factors tested, a peptide designated by us as "ProCell" was identified to possess highest stimulatory activity of HUVEC proliferation. Treatment of both endothelial progenitors and non-purified bone marrow mononuclear cells demonstrated potent induction of endothelial colonies and tube formation. Incubation of bone marrow mononuclear cells with Procell resulted in enhanced ability to stimulate neoangiogenesis in the hindlimb ischemia model and prevent limb loss. Interestingly, localized separate intramuscular administration of ProCell and bone marrow mononuclear cells also resulted in prevention of limb loss. **Conclusions:** ProCell represents a peptide based stimulator of angiogenesis that is potentially useful enhancement of bone marrow mediated angiogenesis in patients with limb ischemia. Given that several FDA cleared devices exist for extraction and isolation of autologous bone marrow, combination of this approach with Procell offers a viable treatment for patients in which angiogenesis is desired.

478. Treatment of Acute Liver Failure Using Cybrocell™ Universal Donor Fibroblast Therapy

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Introduction: CybroCell is an allogeneic preparation of fibroblasts generated under Good Manufacturing Practices (GMP) that has been demonstrated to possess differentiation ability similar to mesenchymal stem cells. Given various types of mesenchymal stem cells are capable of possessing hepatoprotective activities, we sought to determine efficacy of CybroCell in treatment of acute liver failure in the carbon tetrachloride induced model. **Methods**: Acute liver failure (ALI) was induced in C57BL/6 mice by intraperitoneal injection of carbon tetrachloride. CybroCell was intravenously injected (1 million/mouse) 30 min after ALI induction. Hepatic function, pathology, cellular infiltration and cytokine profiles were assessed 2 weeks after the CCl_4 induction.**Results:** CybroCell administration preserved liver function, suppressed fibrosis by reduction of collagen deposition, and inhibited activated hepatic stellate cells up to 2 weeks after administration. Regeneration of hepatic cells was suggested by presence of PCNA positive cells in the liver. Transwell coculturing experiments also showed that CyroCell suppressed inflammatory cytokine production from activated monocytes as well as inhibiting T cell proliferation. **Conclusions:** CybroCell possesses potential to inhibit liver failure through stimulation of hepatic regeneration and suppression of inflammatory processes.

Gene Targeting & Gene Correction II

479. Efficient Gene Targeting in Mouse Zygotes by RNP Electroporation and rAAV Donor Delivery

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Genetically modified mice are crucial for studying disease biology and assessing safety and efficacy of gene therapies. CRISPR/Cas9 ribonucleoprotein (RNP) electroporation of zygotes, or CRISPR-EZ,

has emerged as a simple and high-throughput technique to generate highly efficient and precise genetic modifications in mouse embryos through co-delivery of single-stranded oligo (ssDNA) donors. Here, we explore the combination of CRISPR-EZ with AAV donors to achieve small sequence substitution, targeted gene knock-in, and transgene integration into safe harbor loci in mouse zygotes. Our infectivity assays show that specific natural AAV serotypes efficiently transduce mouse embryos *ex vivo*. When paired with CRISPR-EZ, the rAAV donors mediate precise sequence modification by homology-directed repair (HDR) in up to ~70% of treated zygotes, exceeding the ~40% HDR efficiency observed with ssDNA donors. We also discover that self-complementary AAV donors outperform their single-stranded equivalents, and HDR efficiency depends on the timing of AAV administration relative to RNP delivery. Furthermore, we successfully tagged endogenous loci with a fluorescent reporter, achieving an estimated 20% knock-in efficiency at the blastocyst stage. Currently, we are quantifying the viability of edited embryos and verifying germline transmission of our targeted gene knock-in. We are also applying our approach to integrate whole expression cassettes into safe harbor loci for the generation of transgenic mouse lines. Our findings show that coupling RNP electroporation with AAV donor delivery enables production of reporter mice for gene expression studies and disease models for the development of gene therapies with unprecedented economic and technical ease.

480. Development of an Assay to Detect Pre-Existing Anti-Cas9 Antibodies and an Estimate of the Prevalence of Anti-Staphylococcus- and Streptococcus-Cas9 Antibodies in the US Population

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The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas9 system is a powerful gene editing tool and clinical applications are in the pipeline. Cas proteins are of bacterial origin. Bacterial proteins, previously used in therapeutic interventions, e.g. pseudomonas toxin for targeted cancer therapies, elicit strong immune responses. Moreover, the Cas proteins are derived from Staphylococcus aureus (Sa) and Streptococcus pyogenes (Sp); common human pathogens. A recent report suggests that a very large proportion of the population (79% for Sa-Cas9 and 65% for Sp-Cas9) carries pre-existing anti-Cas antibodies. This finding has legitimately raised fears that these antibodies may limit the effectiveness of the technology. Here, we describe a rigorously developed assay for detecting anti-Cas9 antibodies which is based on procedures and statistical methodologies outlined in industry-authored white-papers and guidance documents from the FDA and EMA. The assay has sensitivities of 0.73 and 0.24 ng/mL for anti-Sa-Cas9 and anti-Sp-Cas9 antibodies respectively and shows minimal cross-reaction between anti-Sa-Cas9 and anti-Sp-Cas9 antibodies. The dynamic range of the assay is maintained in a 1:20 dilution of human serum, which is thus the Minimum Required Dilution, for samples to prevent "matrix interference". A balanced experimental design was executed with 576 measurements on 48 drug naïve serum samples. The results demonstrated that variance in the measurements were due to samples and not due to the analyst, plate/run, plate position or microplate reader. We followed the recommended tiered approach to develop a screening assay and a confirmatory assay for detecting anti-Sa- and Sp-Cas9 antibodies. Statistical analyses for determination of cut-points and assay validation were carried out using a sample size of 48 donors. An independent sample of serum from 200 donors was used to estimate prevalence of anti-Sp-Cas9 and anti-Sa-Cas9 antibodies in the US population. In addition, using a commercial test, we were also able to establish that 58% of these donors have previously been exposed to *Streptococcus pyogenes*. Our confirmatory assay shows that Cas9 specific antibodies occur at frequencies of 5.5% and 1.5% for Sa-Ca9 and Sp-Cas9 respectively. Isotyping of the anti-Cas9 antibodies showed that, 81% IgG1, 9% IgG2, 9% IgG3 for anti-Sa-Cas9 and 33.3% IgG1, 33.3% IgG2, 33.3% IgG3 for anti-Sp-Cas9. We did not find any anti-Sa or anti-Sp Cas9 antibodies that are specific to IgG4. Here, we have provided a robust and useful assay for detecting anti-Cas9 antibodies in drug-naïve subjects and patients treated with Cas9. We show that this assay can detect pre-existing anti-Cas9 antibodies in a small percent of the US population. The clinical relevance of anti-Cas9 antibodies is currently uncertain but the development of tools to identify and interpret antibody responses to Cas9 is a necessary part of bringing Cas9-based therapies to clinical practice.

481. *In Vivo* Comparison of Genome and Base Editing Treatments of a Humanized *Pcsk9* Knock-In Hypercholesterolemic Mouse Model

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The biggest hurdle for therapeutic applications of gene editing is the lack of validated models to assess its safety and efficacy *in vivo*. Wild type mice are not always a valid translatable model to evaluate *in vivo* gene targeting, partly due to their insufficient degree of genomic conservation with man. Proprotein convertase subtilisin/kexin type 9 (PCSK9) targets the low-density lipoprotein receptor (LDL-R) to hepatic degradation, thereby increasing plasma LDL-cholesterol (LDL-C) levels. This makes it a key drug target of innovative biologics like monoclonal antibodies and siRNAs. Here, we aimed at comparing two novel therapeutic approaches based on CRISPR-mediated genomic inactivation of *PCSK9* to validate alternative genetic disruptive therapies to reduce hypercholesterolemia. First, we generated a novel liver-specific human PCSK9 knock-in mouse model (hPCSK9-KI) that develops a human-like hypercholesterolemic phenotype. Then, we used this humanized mouse model to compare *in vivo* genome editing and base editing efficacy. The molecular and physiological editing effects were mediated by specific reduction of either human PCSK9 or murine Pcsk9 protein levels (up to ~ 45%) as well as cholesterol levels (up to

~ 35%). Finally, through targeted deep sequencing, we spotted the differences between genome editing and base editing signatures *in vivo* and we strikingly failed to detect any modifications at predicted off-target sites. Our hPCSK9-KI mouse represents a novel humanized dyslipidemia reporter model that serves as a platform to test therapeutic approaches for reducing human PCSK9 levels. Further, the hPCSK9-KI mouse can be utilized to assess both efficacy and safety of CRISPR-mediated gene therapy strategies *in vivo*.

482. Highly Efficient Transgenesis in Ferrets Using CRISPR/Cas9-Mediated Homology-Independent Insertion at the *ROSA26* Locus

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The domestic ferret (*Mustela putorius furo*) has proven to be a useful species for modeling human genetic and infectious diseases of the lung, neurologic conditions, lung and brain stem cell biology, and eye research. However, biomedical research in ferrets has been hindered by the lack of rapid cost-effective methods of genome engineering. Here, we utilized CRISPR/Cas9-mediated homology-independent non-homologous end joining (NHEJ) in zygotes to create ferrets that carry a dual-fluorescent Cre-reporter system flanked by PhiC31 and Bxb1 integrase *att*P sites at the *Rosa26* "safe harbor" locus. Ferret zygotes were generated and co-injected with a donor plasmid and ribonuclear protein (RNP) complex composed of SpCas9 protein and the *ROSA26*-sgRNA. Concurrent digestion of the *ROSA26* genomic locus and the donor plasmid DNA by the Cas9/*ROSA26*-sgRNA RNP resulted in highly efficient and site-specific integration of the transgene via homology-independent repair. In total, 179 zygotes were injection of which 151 surviving embryos (84%) were transplanted into 5 pseudopregnant jills. Of the 23 live births, 6 (26%) demonstrated expression of the tdTomato transgene as judged by fluorescent body scans. Two of the six transgenic kits died shortly after birth due to poor jill care. The encoded tdTomato transgene was highly expressed in all tissues evaluated. Targeted integration was verified in the four surviving founders by PCR analyses, Southern blot, and germ-line transmission. Three of the four founders contained insertions in the reverse directly of ROSA26 intron 1, as designed using self-inactivating sgRNA sites in the plasmid and genome. Insertion in the fourth founder was in the forward orientation. One of the four founders contained the backbone of the plasmid within the insertion site and two founders also had random trasngene integration events at a second locus. Function of the *ROSA26*-CAG-^{Lox}tdTomat^{LoxStop}-EGFP (ROSA-TG) Cre-reporter was confirmed in primary cells following Cre expression. The construction of Cre-driver ferrets for specific lung stem cell lineages are under construction for use with this *ROSA26*-Cre reporter ferret. Additionally, the Phi31 and Bxb1 integrase *att*P sites flanking the transgene can also be used for rapid directional insertion of any transgene at the *ROSA26* locus. These methods and the ferret model generated will greatly enhance applications of lineage-tracing, evaluation of stem cell therapies, and transgenesis in ferrets for biomedical research.

483. Targeting the Hemoglobin Loci in Hematopoietic Stem Cells for Systemic Protein Expression

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Most genome editing strategies aim to correct diseases by repairing underlying mutations at their genomic loci; however, the limited correction efficiency and the challenge of tailoring a different approach for each gene mutation may hamper their clinical translation.To address these issues, we developed a novel platform for hematopoietic stem/progenitor cells (HSPCs) based systemic protein expression. We took advantage of the CRISPR/SpCas9 system to insert different transgenes under the control of the α and β-globin promoters in primary HSPCs in order to redirect a fraction of the exceptional hemoglobin synthesis capacity of HSPCs-derived erythroid precursors (~7.2gr/day) for the production of therapeutic proteins. Since hemoglobin genes are expressed only in the erythroid lineage, they are not required for cell survival of non-erythroid cells, and the disruption of 2 of the 4 α-globin or 1 of the 2 β-globin alleles has no functional consequences, they represent a safe and advantageous genomic locus for transgene integration.To this aim, we first identified several gRNAs targeting non-coding regions of α and β-globin genes with few predicted off-targets and high on-target activity in fetal erythroid K562 cells. Using integration defective lentiviral vectors, we inserted different promoterless GFP donor cassettes at gRNAs cutting sites and we demonstrated proper and functional GFP integration by PCR and flow cytometry on both K562 bulk population and single cell clones. For the selected α-globin gRNA we further confirmed these results using HUDEP2 cells -an adult erythroid cell line- and replaced GFP with additional human transgenes, including human FVIII and FIX (measured by ELISA and aPTT assay).We then moved to clinically relevant HSPCs, where we confirmed high on-target activity of the selected α-globin gRNA when delivered as Cas9 ribonucleoprotein in HSPCs ex vivo (64% ± 8.5 of InDels). In addition, we optimized targeted integration conditions using an AAV6 vector encoding a promoterless GFP cassette flanked by homology arms for the gRNA site and we achieved up to 58% of on-target events, as measured by flow cytometry and digital droplet PCR. Notably GFP expression was restricted to the erythroid lineage, as only red colonies scored positive in colony-forming cell (CFC) assay, and recapitulated hemoglobin expression patterns during HSPCs erythroid differentiation. Although we achieved high level of genome modification, this approach was safe since globin synthesis was unchanged in edited HSPCs-derived erythroblasts, as assessed by HPLC analyses of single globin chains and hemoglobin tetramers in both liquid culture and CFC. Finally, we will have data regarding targeting of therapeutic transgenes in HSPCs and unbiased genome wide analysis of Cas9/gRNA nuclease specificity.In summary,

we established a safe and novel ex vivo nuclease-based platform for robust expression of therapeutic transgenes for the treatment of genetic and non-genetic diseases.

484. Engineered High Specificity Cytidine Deaminase Enables Single Nucleotide CRISPR-Cas9 Base Editing

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Most genome editing reagents operate by introducing DNA double strand breaks (DSBs) at a specified genomic target site and rely on the endogenous DNA repair pathways to determine the outcome. This strategy often results in insertions, deletions, or translocations, all of which limit user-defined repair outcomes. CRISPR-Cas9-based base editor (BE) technology allows users to create precise genomic C to T mutations without the need to introduce DSBs and without an endogenous or user-provided template, overcoming much of the inherent stochasticity associated with DSB-based editing. However, current generation BEs lack the resolution to selectively modify single nucleotides within an approximately 5 base pair editing window, a critical parameter to avoid deleterious bystander mutations when targeting coding sequences or gene regulatory elements. Here, we describe a sequence-specific, high fidelity C-to-T base editor that exhibits activities comparable to BE3 on target cytidines preceded by a 5' thymidine, but greatly reduced or absent editing on cytidines in other sequence contexts. Strikingly, this engineered protein also has excellent genome-wide specificity compared to BE3, even with sgRNAs targeted to homopolymeric sites in human cells. Together, these properties allow for efficient and highly specific single nucleotide base editing in human cells, including of disease relevant loci.

485. HDAd5/35++ Vector Expressing Anti-CRISPR Peptides Controls the Duration of CRISPR/Cas9 Activity and Decreases CRISPR/Cas9-Associated Toxicity in Human Hematopoietic Stem Cells

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We developed helper-dependent HDAd5/35++ vectors expressing CRISPR/Cas9 for potential hematopoietic stem cell (HSC) gene therapy of β-thalassemia and sickle cell disease (HDAd-CRISPR). The HDAd-CRISPRs are targeted to genomic sites involved in the suppression of (fetal) γ-globin. The process of CRISPR/Cas9 gene transfer using these vectors did not cause acute apoptosis in CD34+ cells and did not affect their in vitro expansion and erythroid differentiation. However, functional assays for primitive HSCs, e.g. multilineage progenitor colony formation and engraftment in irradiated NSG mice, revealed toxicity of HDAd-CRISPR vectors. HDAd5/35++ vectors expressing sgRNA without homology in the human genome also displayed toxicity (although to a lesser degree than HDAd-CRISPR vectors) indicating that, in addition to CRISPR/Cas9-mediated double-strand DNA breaks, the Cas9/sgRNA complex has other detrimental effects on HSCs. To control CRISPR/Cas9 activity, we generated a HDAd5/35++ vector that expressed two anti-CRISPR (Acr) peptides (AcrII4 and AcrII2) capable of binding to the Cas9/sgRNA complex (HDAd-Acr). Timed transduction with the HDAd-Acr vector after completion of Cas9-sgRNA-based gene editing decreased cytotoxicity to HSCs. CD34+ cells that were sequentially infected with HDAd-CRISPR and HDAd-Acr (with an interval of 2 days) engrafted at a significantly higher rate. In contrast to transduction without HDAd-Acr, the frequency of CD34+ cells in engrafted human cells was not decreased. Target site disruption frequencies in engrafted human cells, measured 10 weeks after transplantation, were similar to those in pre-transplantation CD34+ cells indicating that gene edited primitive HSCs survived. In vitro differentiated HSCs isolated from transplanted mice demonstrated increased γ-globin expression as a result of gene editing. Our data indicate that the HDAd-Acr vector can be used as a tool to reduce HSC cytotoxicity of the Cas9/sgRNA complex.

486. AAVHSC Nuclease-Free Genome Editing Leads to *In Vivo* Genome Correction and a Significant Reduction in Disease Phenotype in a Mouse Model of Phenylketonuria

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The direct correction of pathogenic mutations has great potential for the treatment of genetic disorders. A novel group of Clade F adeno-associated viruses has been isolated from normal human CD34+ hematopoietic stem cells (AAVHSCs) and has shown high-efficiency nuclease-free gene editing as well as gene transfer capabilities, and here we set out to further explore the potential of AAVHSCs for *in vivo* gene editing. To measure the efficiency of *in vivo* editing, AAVHSC capsids were packaged with a construct containing a promoterless luciferase cassette flanked by sequences homologous to the 7th intron of mouse F8 (AAVHSC-F8Luc). Intravenous administration of AAVHSC-F8Luc into NOD/SCID mice resulted in liver-specific expression of luciferase that was maintained for up to 9 weeks. Genotyping analysis of genomic DNA derived from the livers of treated mice display a significant proportion of F8 alleles were successfully edited, harboring the luciferase cDNA in the F8 intron. In addition, next generation sequencing of the integration site confirmed precise editing with no detection of indels or ITR integration. To investigate the therapeutic potential of the platform to correct a disease phenotype, an AAVHSC correction vector (AAVHSC-PAH) containing a promoterless cDNA encoding human phenylalanine hydroxylase (*PAH*) flanked by sequences homologous to exon 1 of the murine PAH gene was used to treat ENU2 mice, a commonly used model of the disease phenylketonuria (PKU). Intravenous injection of AAVHSC-PAH led to a statistically significant reduction ($p<0.001$ at all time points) in serum Phe levels (PKU disease marker) from baseline and compared to untreated controls. This correction was maintained over 21 weeks

post injection. Genotyping analysis of genomic DNA derived from the livers of treated mice display efficient gene correction, due to the integration of human PAH into exon 1 of the murine PAH gene. Furthermore, in-situ hybridization of mRNA with probes targeting human PAH were observed throughout the liver. In total, these data provide evidence that the AAVHSC genome editing platform is capable of *in vivo* genetic and phenotypic correction at therapeutically relevant levels by precise forms of DNA recombination.

487. Self Cleaving guideRNAs for Selective Expansion of Precisely Gene Edited Hepatocytes In Vivo

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Recombinant adeno-associated viruses (rAAVs) are promising for use in liver targeted human gene therapy. For the treatment of genetic disorders, life-long, physiologically regulated expression of therapeutic transgene after a single gene therapy treatment would be desirable. However, standard rAAVs remain mostly episomal and are therefore lost during cell replication, including during normal growth in children. One method to address the limitations of the current technology is to directly repair the mutation in hepatocytes. However, the efficiency of *in vivo* gene repair with methods available at the present time is very low. One strategy to achieve higher efficiency of gene editing is to select cells that have acquired the desired targeting event. This can be achieved by linking the desired modification to a selectable gene disruption in cis, such that the selection can happen only if proper gene targeting has been achieved. We have previously shown that shRNAs embedded in a microRNA within an intron of a liver gene can generate gene knockdown after gene targeting using a human Factor 9 (hF9) expressing Generide AAV vector. Expression of the shRNA was driven by the strong albumin promoter. While this approach certainly can work, shRNA's usually only knock down a gene by approximately 90%. This incomplete inhibition of the protective gene limits the potential application of novel in vivo drug selection regimens and restricts gene targeting to only loci with very strong transcriptional activity, capable of generating sufficient shRNA levels. In contrast, complete gene knockouts can be achieved by CRISPR-cas9 cutting. In order to generate selectable CRISPR-mediated gene knockouts, it is necessary to restrict its endonuclease activity to only cells that have proper gene targeting. This could be achieved if the guide-strand RNA (gRNA) is expressed only after proper homologous recombination. This strategy requires that the gRNA expression is driven by a tissue-specific polymerase 2 promoter. Here we show that flanking RNAs with self-cleaving ribozymes permits the proper processing of active gRNA from polymerase 2 transcripts. In $Fah^{-/-}$ mice, loss of *Hpd* or *Hgd,* genes upstream of FAH in the tyrosine degradation pathway, provides a selective advantage for hepatocytes. We observed strong in vivo selection of albumin targeted hF9 gene ride vectors harboring self-cleaving gRNA (scgRNA) against *Hpd* and *Hgd*. Human F9 dramatically increased in $Fah^{-/-}$ mice treated with these vectors and then taken off NTBC, achieving superphysiologic levels. In order to broaden this paradigm, we have now engineered a system that renders hepatocytes resistant to the widely used drug acetaminophen. A scgRNA designed to knock out NADPH-cytochrome P450 reductase (Cypor) was incorporated into the albumin targeted hF9 gene ride vector. We show that loss of Cypor prevents cytochrome p450 enzymes converting acetaminophen into its toxic metabolite. Acetaminophen treatment resulted in the expansion of the Cypor null hepatocytes to approximately 45% of the liver mass. Experiments measuring hF9 expression in response to acetaminophen treatment are ongoing.

488. A Precise Tool to Determine and Optimize Efficiency of Different DNA Double Strand Break Repair Outcomes in Hematopoietic Stem and Progenitor Cells

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Gene editing in hematopoietic stem cells (HSC) can permanently correct a variety of hematological disorders. However, the double-strand breaks (DSB) caused by different site-specific nucleases are predominantly repaired by the 'error-prone' non-homologous end-joining (NHEJ) DNA double-strand break repair (DDR) pathway causing gene disruption, and only a minor fraction repaired precisely via homology-directed repair (HDR). Improving HDR has wide implications correction of pathological mutations. Also, DDR via the microhomology-mediated end joining (MMEJ) can be extremely useful in removing small regulatory elements for therapeutic use, especially for hemoglobinopathies. However, editing the rare HSC amidst the CD34+ hematopoietic stem and progenitor cell (HSPC) population is particularly challenging, since HSC comprise 1-2% of HSPC, and do not withstand prolonged culture for selection of the few HDR/MMEJ events. Currently, the common readout of the edited HSC is by molecular analysis of total CD34+ population. Gantry et al (Cell Reports, 2017) recently showed an elegant ko of the CD45 surface reporter by CRISPR/Cas9 to detect NHEJ outcomes in HSPC by FACS. We identified a unique gRNA location in the human CD45 gene that would rapidly and precisely quantify NHEJ, HDR in the human HSC/HSPC populations using a FACS at a single cell level and MMEJ via TIDE assay; and designed a HDR donor template that would also detect random integration (RI) events. The gRNA/Cas9 induced DSB was repaired primarily via a 1bp insertion and an occasional 2 and 4bp deletion, causing a frame-shift and detection of NHEJ DDR 100% of the time at a single cell/allele level, by either knocking out CD45 expression (bi-allelic NHEJ) or reducing CD45 expression (mono-allelic NHEJ). In addition, this gRNA cut site was flanked by 3bp microhomology, resulting in a 6bp in-frame MMEJ, detected by TIDE assay specifically in the CD45+ population. For HDR and RI, the donor template had an in-frame promoterless GFP with a tNGFR expression cassette downstream of the 3' homology domain (GFP/tNGFR donor). EBV immortalized B lymphocytes (EBL) that repaired the DSB via NHEJ on a single allele had low CD45 expression, while bi-allelic DDR via NHEJ resulted in CD45 KO, detectable by FACS. Using this rapid reporter, we optimized >95% biallelic CD45 KO in EBL, and >80% CD45 KO in CD34+ HSPC and CD34+38-90+RA-49f+ HSC, and confirmed the precision of the FACS detection by TIDE assay. Edited HSPC resulted in 45±10% total engraftment and 39±16% edited cell engraftment (n=12) in NSG mice. TIDE assay showed that 10-15% CD34+ HSPC repaired

gRNA/Cas9 induced DSB via MMEJ via in frame 6bp deletion. Similar levels of MMEJ was detectable in myeloid cell lines, but not in EBL. We were able to reduce MMEJ via small molecule inhibitors of the MMEJ DDR, and completely block MMEJ via siPOLQ, a key enzyme in the MMEJ pathway, and solely cause NHEJ DDR. Conversely, MMEJ could be increased by siRad51, a molecule that competes with POLQ for end-resection. Furthermore, provision of the GFP/tNGFR HDR donor along with gRNA/Cas9 resulted in HDR, detected at a single cell, single allele level (CD45+GFP+ cells), optimized to 30-50% in hematopoietic cell lines, with a corresponding reduction in NHEJ DDR; Optimization of HDR in HSPC/HSC is underway and will be presented. Additionally, RI of HDR donor, seen as tNGFR+ cells. Using this precise tool, we are optimizing MMEJ. Increased HbF expression, by editing the HPFH locus and Bcl11a enhancer via MMEJ DDR-mediated deletion of key regulatory sequences can potentially treat both β-thalassemia and sickle cell anemia and HDR. In summary, we have designed a rapid precise reporter that allows determination of, and optimization of MMEJ, HDR, NHEJ DDR and RI after site-specific nuclease-mediated DSB in HSPC and HSC, allowing rapid optimization of HSC editing.

489. Correction of Multiple Cystic Fibrosis-Causing Variants by CFTR Superexon Homology-Independent Targeted Integration (HITI)

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Cystic Fibrosis (CF) is caused by mutations in both alleles of the CFTR gene. Whilst 90% of individuals with CF carry at least one allele with F508del there are more than 270 other disease-causing variants. The use gene editing to correct the F508del allele has been reported in cell and animal models, but the efficiency of this template-driven homology-directed repair (HDR) approach is rarely above 1% of transfected cells without drug selection (McNeer, 2015; Hollywood, 2016). The use of a superexon donor containing fused exons 11-27 as HDR template has establish the proof-of-principle for correction of multiple mutations (Bednarski, 2016) but the efficiency was even lower. Much higher gene editing efficiency can be achieved by exploiting the non-homologous end joining (NHEJ) DNA repair pathway; we have recently show targeted excision of CF-causing deep intronic mutations up to 40% of transfected cells (Sanz et al., 2017). Here we describe the incorporation of two different superexons into the CFTR locus using the NHEJ-based pathway by HITI (homology-independent targeted integration). HITI results in the physical integration of a foreign DNA at a Cas9-induced double strand break in genomic DNA, and shows significantly higher levels of gene-editing efficiency compared to HDR based techniques (Suzuki et al., 2016). HITI also has the advantage over other NHEJ based integration techniques in that it prevents integration of foreign DNA in the wrong orientation, and works very efficiently in slow/non-dividing cells such as long-lasting lung epithelial cells that are potential target cells for gene editing in individuals with CF. The superexons comprises an inverted gRNA target site, a splice acceptor site and either CF exons 11-27 or 23-27 fused as a partial cDNA linked to the 2A-mCherry reporter, we have observed targeted integration into intron 10 or 22 respectively using Cas9/gRNAs previously validated in our lab (Sanz, 2017). Analysis of the 5' and 3' integration sites shows a high level of precise integration. Successful integration of the CF Superexon 11-27 or 23-27 should result in the expression of mRNA with wild-type sequence that can potentially correct 92% or 5% of known CFTR variants respectively. Chimeric mRNAs will be expressed under most of normal regulatory features of the intact gene, including the promoter and the intronic regions upstream to the integration site, which stays unaltered. Although integration of the Superexon sequences has been successful, initial attempts to characterize the chimeric mRNAs containing CFTR-mCherry sequences derived from the integrated superexons have been difficult due to sequence identity between the endogenous transcript and the superexon-derived transcript. To address this, we have designed modified superexon sequences which contain unique primer-specific sequences to enable detection by RT-PCR (see Bednarski et al., 2016) and/or short in-frame insertions to be detected by RT-PCR and FLA analysis. These variant superexons will be used to characterise the restoration of normal splicing and subsequent functional rescue of CF mutations in suitable human cell models.

490. Expression of CRISPR-Cas9 Single Guide RNA's Using Small tRNA Promoters

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The CRISPR-Cas9 DNA editing system has emerged as a powerful tool for the targeting and mutagenesis of specific DNA sequences. The *in vivo* applications of CRISPR-Cas9 will likely require the use of adeno-associated virus (AAV)-based viral vectors due to their lack of pathogenicity in humans and their ability to be produced at very high viral titers. Unfortunately, AAV vectors are capable of packaging only a modest ~4.7 kb of DNA sequence which is problematic given the large size of the Cas9 protein and the pol III based cassette necessary for single guide RNA (sgRNA) expression. Much work has been done in order to identify and characterize highly active Cas9 proteins that are significantly smaller than the prototypical *Streptococcus pyogenes* Cas9 protein. However, little innovation has been described with regard to novel sgRNA expression methods that are more compact that the typical ~250 bp U6 promoter expression cassette. Here, we report that small, ~70 bp tRNA promoters of human and viral origin can be used to express high levels of tRNA:sgRNA fusion transcripts that are efficiently cleaved by endogenous tRNase Z to release fully functional sgRNAs. This work has the potential to greatly facilitate the construction of highly effective CRISPR-Cas9 based AAV vectors for *in vivo* studies and medical interventions.

491. Programmable Exon Skipping with Adenine Base Editors

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Exon skipping has been shown to be an effective treatment for diseases such as epidermolysis bullosa, cancer, frontotemporal dementia,

inflammatory diseases, spinal muscular atrophy and even Duchenne muscular dystrophy, where mutations in coding exons lead to non-functional gene products. One potential treatment for these diseases is skipping of the exons containing mutations, which often results in expression of protein isoforms that are shorter but retain partial functionality and can effectively correct the disease. Exon skipping has been traditionally accomplished with antisense oligonucleotides (AONs), which modulate pre-mRNA splicing by hybridizing to a target sequence to prevent the binding of spliceosome machinery. However, since the effects of AONs are only temporary, there is a pressing need for technologies that enable permanent exon skipping. More recently, CRISPR-Cas9 gene editing has been used for inducing exon skipping by introducing double-strand breaks (DSBs) within exons or splice acceptors, which, when repaired by non-homologous end joining, can result in exon skipping. However, a major limitation of gene editing techniques that rely on DSBs is that the repair process creates stochastic mutations with unpredictable phenotypic consequences. Alternatively, single base editors circumvent this problem by introducing C>T or A>G nucleotide conversions at targeted sites without introducing DSBs. Our studies support that adenine base editors can be used for introducing A>G mutations within splice acceptors, which lead to exon skipping. In this work, we demonstrate that this method is broadly applicable for programmable splicing in mammalian genomes by targeting multiple exons in different genes across a panel of cell lines. We used different types of assays to detect and quantify exon skipping, including PCR, Sanger sequencing, and deep sequencing. We observed that the rate of exon skipping increased progressively over time and submaximal levels were not detected until six days after gene delivery. Our results demonstrate a correlation between base editing in genomic DNA and rate of exon skipping in RNA. It is also noteworthy that alternative splicing efficiency depended critically on the ratio of sgRNA to base editor plasmids delivered to the cell. Since modification rates in our pilot experiments were low, we optimized base editing activity by systematically modifying the length of the linker tethering the adenosine deaminase and Cas9 nickase. These experiments yielded a collection of base editors that exhibit a wide range of activity at different target sites. Finally, we anticipate that further development of adenine base editing technology, such as packaging into adeno-associated viruses or additional protein engineering focused on increasing editing efficiency and expanding the range of exons that can be targeted, will enable diverse therapeutic applications based on exon skipping.

492. Engineering a *BTK* Targeting Megatal for Clinical Gene Modification

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MegaTALs are monomeric hybrid nucleases comprising a transcription activator-like effector (TALE) DNA binding domain linked to a homing endonuclease (HE) cleavage domain. The cleavage editing efficiency and specificity properties of megaTALs make them attractive reagents for clinical gene editing applications. However, the optimal strategies for megaTAL engineering and their relative performance in genome modification applications have not been rigorously explored. In the current study, we engineered multiple HE I-OnuI variants targeted to recognize a unique site in the human Bruton's tyrosine kinase (*BTK*) gene (a gene mutated in the immunodeficiency disease X-linked agammaglobulinemia) using yeast surface display (YSD). These BTK-I-OnuI variants were combined with a TALE DNA binding domain containing an 11-repeat variable di-residue (RVD) array recognizing a corresponding 11 base pairs upstream of the HE target site. The editing efficiency of this series of BTK HE and megaTAL enzymes was further evaluated in a mammalian cell line reporter system, and selected megaTALs were tested in primary human CD4+ T cells and compared with previously characterized BTK TALENs. Here, we demonstrate that BTK HE binding affinity correlates with editing efficiency in the absence of a TALE array, however cleavage efficiency of the BTK HE using the YSD system is a better predictor of BTK megaTAL editing efficiency. In primary human CD4+ T cells, BTK HE enzymes with balanced YSD cleavage efficiency and binding affinity, but not with the highest BTK HE cleavage efficiency, exhibited the highest editing efficiencies as megaTALs. Both the BTK megaTAL and the BTK TALEN achieved near complete editing (>90% indels) of the target site, with megaTALs having improved viability following delivery using mRNA transfection. In the presence of donor AAV template, both enzymes induced high efficiency homology directed repair (HDR) in human primary T cells. Based on these observations, we have modified our engineering strategy to select for HE variants with balanced cleavage efficiency in the YSD system and binding affinity, rather than reagents with the highest HE cleavage efficiency. This modification significantly improved the generation of highly efficient megaTALs, and should facilitate rapid development of new reagents for additional clinical genome editing applications.

493. Streamlined Production, Application, and Analysis of Pooled Genome-Wide sgRNA Lentiviral Libraries

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Genome-wide loss-of-function genetic screens are a powerful way to identify novel protein functions and biological processes within a cell. A common approach among *in-vitro* loss-of-function screens involves knocking out genes in a population of cells, applying selective pressure, and then identifying mutations that are either enriched or depleted in the selected population relative to a control. The easy programmability and high knockout efficiency of the CRISPR/Cas9 system has helped researchers maximize the potential of this *in-vitro* screening method to identify genes responsible for a given phenotype of interest. Current methods used to apply pooled sgRNAs in loss-of-function screens rely on the efficiency and permanence of lentiviral vector-based delivery, and typically employ next-generation sequencing to analyze the resulting distribution of sgRNA sequences in screened cell populations. Inherent challenges include maintaining sgRNA representation in the plasmids used to produce the lentivirus, achieving an optimal titer upon scale-up of lentivirus production, and preparing high-quality NGS

libraries that accurately reflect the distribution of sgRNA sequences in the resulting cell populations. Here we present streamlined methods for producing Cas9+/sgRNA+ cell populations in sufficient quantities for a genome-wide screen, and for generating NGS libraries used to assess changes in sgRNA representation post-screen. Our approach for producing a high titer, sgRNA lentivirus library consists of a single-tube format in which all the elements required for sgRNA library production are lyophilized, including the lentiviral packaging mix and polymeric transfection reagent. Only water needs to be added to produce a transfection mix capable of transfecting 293T cells for high-titer virus production. The second-generation sgRNA library employed by our method contains highly active, on-target sgRNAs chosen from the Brunello sgRNA library, targeting 19,114 genes with 76,610 unique guide sequences, and is pre-validated for sgRNA representation before application to the lyophilized formulation. Production of Cas9-expressing lentivirus is achieved in a similar fashion. As a proof of concept, we used this system to produce a Cas9+/sgRNA+ A375 cell population from which random clones were chosen, corresponding sgRNA sequences were identified, and cleavage of genomic targets was assessed by resolvase assay. In all clones analyzed, sgRNAs were found to be active and capable of producing indels at the intended target site. To further demonstrate the activity of this pooled library, we performed a screen for resistance to the purine analog 6-thioguanine and were able to identify the purine salvage pathway genes HPRT and NUDT5. Each gene was represented in the selected population by four corresponding sgRNA sequences, providing internal validation for the assay and for each gene's activity in this pathway. In addition, the sgRNAs targeting HPRT were enriched by several orders of magnitude relative to their frequencies in the starting population. Our results are consistent with those published previously using the Brunello sgRNA library. For analysis of the screened populations, we wanted to avoid issues with PCR primer design, gDNA isolation, and amplification of sgRNA sequences, so we developed a complimentary workflow for gDNA purification and sgRNA sequence amplification for NGS library construction to be used with this sgRNA library system. The pairing of a simplified, high-efficiency method for creating Cas9+/sgRNA+ cell populations with optimized reagents for NGS analysis should enable novice users to perform genome-wide phenotypic screens without concerns regarding sgRNA representation, low-titer virus production, or NGS library preparation.

494. Small Molecule Enhancement of Genome Editing

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While hematopoietic stem and progenitor $CD34^+$ cells (HSPCs) have promising applications in clinical therapies, genome editing of these cells has proven to be challenging, costly and time-consuming. Achieving sufficient targeting efficiencies in HSPCs via conventional, non-viral methods, continues to be an insurmountable task. However, small molecules may offer a solution to this persisting problem. These molecules include a variety of synthetic and natural compounds capable of influencing diverse cellular processes, which may offer benefits in gene therapy. We hypothesize that the treatment of HSPCs with small molecules may enhance the overall efficiency of homology driven recombination mediating targeted integration (TI) of transgenes. As a preliminary part of our investigation, we analyzed the effects of four small molecules on the overall efficiency of genome editing using two nuclease platforms: the CRISPR/Cas9 system and the bioengineered nuclease, TALENs. K562 and Jurkat cells were each treated with one of the small molecules prior to nucleofection with a plasmid encoding the engineered nuclease along with a second plasmid encoding a donor DNA template. Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, was confirmed to demonstrate enhancement. K562 cells demonstrated an enhanced efficiency of TALENs and CRISPR/Cas9-mediated TI as evidenced by a ~30% and ~25% respective increase in reporter GFP expression. Treatment with butyrate, another HDAC inhibitor, also led to a substantial increase (2-fold) in the expression of reporter GFP in K562 cells, indicative of additional TIs. We considered a third small molecule, metformin, because of its well-documented, synergistic effects when used in conjunction with VPA during the treatment of renal cell carcinoma (Zhang et. al 2015). Treatment of K562 cells with metformin alone also yielded a pronounced improvement in the efficiency of CRISPR/Cas9-mediated genome editing. Reporter GFP expression of metformin-treated cells exceeded that of control cells by up to 3-fold, achieving as high as 98% targeting in K562 cells. Combined exposure to VPA and metformin did not yield enhancements in TI efficiency beyond those observed after the treatment of cells with metformin alone. In addition, we analyzed the effects of a fourth small molecule, 2,3-DCPE hydrochloride. Since this compound is an S phase inhibitor and homology directed repair is thought to be most active during this phase, a higher level of TIs was expected following 2,3-DCPE hydrochloride treatment. However, we failed to observe any significant improvements in genome editing efficiency. Each of the compounds, including 2,3-DCPE hydrochloride, yielded a dose-response relationship in the cell lines with treatments of higher concentrations leading to decreased levels of cell viability. We observed the most consistent and significant improvements in overall TI efficiency when using VPA and metformin individually, both of which are already FDA-approved. Similar enhancements with these compounds in regards to genome editing efficiency were observed in HSPCs. As HSPCs have the potential to fully repopulate the bone marrow and blood, enhanced genome editing in these cells could lead to significant advances in the treatment of several hematologic diseases.

495. Cas13 Targeting of the UBE3A-ATS RNA for Treatment of Angelman Syndrome

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New advances in gene editing technologies have paved the way for the development of gene therapies that were not feasible in the past. Angelman Syndrome is a neurodevelopmental disorder caused by deletion of the gene UBE3A, which is paternally imprinted in neurons by UBE3A-ATS, a long-noncoding anti-sense transcript. Due to this imprinting, patients lack an expressed copy of UBE3A leading to disease phenotypes. Therapeutic development for Angelman has focused on ways to reactivate the silenced paternal allele of UBE3A, but efficient methods are yet unproven. The recent characterization of Cas13, an RNA-specific nuclease that results in degradation of the target transcript, represents a possible avenue for the delivery of a

therapeutic that can persistently target a transcript rather than directly editing the DNA genome. Cleavage of the UBE3A-ATS should restore expression of UBE3A. We designed variants LwaCas13a and PspCas13b to target sites along the UBE3A-ATS in human neuronal cell models and measured its degradation and resulting impact on the expression of UBE3A. Transcriptome editing via Cas13 may prove to be a more viable therapeutic method for gene silencing than other tradition methods utilizing shRNA or anti-sense oligonucleotides.

496. *In Vitro* Gene Editing Catalyzed by CRISPR-Cpf1 and a Mammalian Cell-Free Extract

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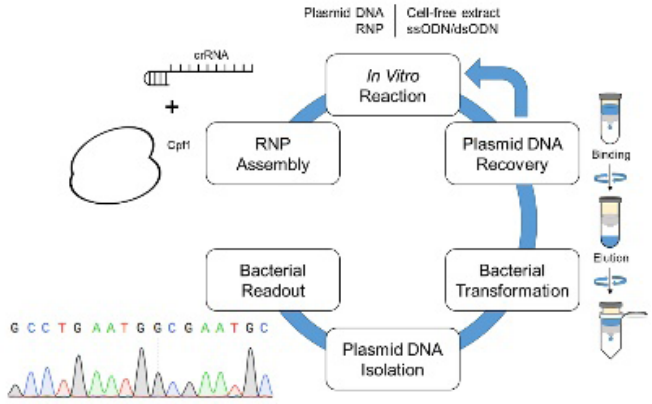
Extraordinary efforts are underway to offer greater versatility and broader applications for CRISPR-directed gene editing. Here, we report the establishment of a system for studying this process in a mammalian cell-free extract. A ribonucleoprotein (RNP) particle and a mammalian cell-free extract coupled with genetic readout are used to generate and identify specific deletions or insertions within a plasmid target. A Cpf1 RNP induces a double-stranded break and the cell-free extract provides the appropriate enzymatic activities to direct specific deletion through resection and homology directed repair in the presence of a single-stranded and double-stranded donor DNA fragment. Site-specific deletions through DNA resection and site-specific insertion of appropriate donor DNA fragments are enabled in the same reaction mixture.



These reactions could recapitulate the competing pathways of nonhomologous end joining (NHEJ) and homology directed repair (HDR). A heterogeneous population of plasmid molecules containing deletions, specific insertions or other forms of genetic modifications are generated in a single *in vitro* gene editing reaction mixture. The development of this system provides an opportunity to study the molecular interactions and the regulatory circuitry controlling CRISPR-directed gene editing in a more defined manner. This cell-free system establishes a foundation to study the heterogeneous products of gene editing as well as the relationship between nonhomologous end joining and homology directed repair and related regulatory pathways simultaneously in a controlled environment. We also demonstrate that this cell-free system can be used for site-specific mutagenesis in the absence of PCR. The reaction is comprised of the same components used for site-specific DNA insertion but relies on adjacent cleavage sites catalyzed by two Cpf1 RNPs and double-stranded DNA donor fragments with short arms of DNA homology. We first demonstrate the mechanics of a gene segment replacement reaction using a fragment of the lacZ gene then demonstrate the utility of the approach for site-specific mutagenesis using KRAS as the target gene. Insertion of the donor fragment bearing the altered bases is catalyzed totally by the mammalian cell-free extract.

497. Transient Epigenetic Gene Therapy as a Hit-And-Run Targeted Epigenome Editing Approach Driven by Nuclease-Null CRISPR-Dcas9 for Age-Related Disorders and Degenerative Diseases

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Down-regulation of endogenous genes essential to the regenerative capacity of relevant tissue-specific stem cells is a common age-related feature (e.g. SIRT3 in hematopoietic stem cells, NAMPT in bone marrow mesenchymal stem cells). Epigenetic reactivation of such endogenous stem cell genes stands thus as a potential "rejuvenating" strategy for age-related disorders/degenerative diseases and is one of the potential applications of the transient epigenetic arm of our proposed Universal Stem Cell Gene Therapy platform. Based on a hit-and-run protocol, it is aimed at long-term transcriptional gene silencing/activation through the transient action of epigenetic/epigenomic effectors. Initially based on promoter/enhancer-specific siRNAs or short sense/antisense RNAs/oligonucleotides, it culminates now with double-mutant nuclease-null CRISPR-dCas9 as a breakthrough programmable sgRNA-guided DNA-binding module for targeted delivery of fused epigenetic effector domains such as DNA methylases/demethylases. Indeed, directed to its ~20bp genome target by an easily customizable short sgRNA, dCas9 has been shown by others to efficiently drive 1) the TET1 catalytic domain and DNMT3a one for targeted demethylation and methylation of CpG islands from specific gene promoters/enhancers, respectively and 2) the catalytic core of the human histone acetyltransferase p300 and of the KRAB repressor for transcriptional gene activation and silencing via post-translational histone modifications, respectively. Easy and low-cost genesis of custom sgRNAs culminates now in synergistic combinations involving up to three epigenetic effectors, thereby providing the means to fully implement the hit-and-run principle of transient epigenetic gene therapy/epigenome editing (stability through cell division) even in the case of difficult-to-handle genes or/and cells. Unlike small molecule effectors such as 5-aza-2'-deoxycytidine or trichostatin A, gene activation/silencing mediated by dCas9 fusion proteins is highly specific, sharply confined to the targeted gene without spreading to nearby genes, thereby freeing such an epigenome editing technique from off-target safety hazards. Our current age-related focus is the NAD+ salvage pathway, a major aging target. Age-related pathologic down-regulation of its key nicotinamide phosphoribosyl transferase (NAMPT) enzyme seems to be correlated to hypermethylation of the main CpG island of its circadian-tuned NAMPT gene. NAMPT,

nicotinamide mononucleotide adenylyltransferase-1 (NMNAT1) and NMNAT3 transgenes are already involved in pre-clinical gene therapy protocols. Based on ex vivo hit-and-run protocols and mediated by genome-safe delivery of therapeutic mRNAs and sgRNAs (RNA nanoparticules or RNA nucleofection), our approach aimed at curing pathologic age-related NAD+ deficiencies is discussed in light of 1) current pre-clinical in vivo/ex vivo gene therapy experiments based on AAV vectors, 2) of ex vivo stem cell treatment with rejuvenating and senolytic factors, 3) of the potential use of synergistic combinations of activating and silencing epigenetic effectors directed at a set of genes, culminating possibly in stem cell conversions, 4) of the current microRNA technology and 5) of the choice of the autologous stem cells based on the age-related target diseases.

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498. CRISPR-Mediated Targeted Insertion of *Cybb* cDNAs into the *Cybb* Locus for Correction of X-CGD Patient CD34⁺ Cells

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X-linked chronic granulomatous disease (X-CGD) is an immune deficiency characterized by defective production of microbicidal reactive oxygen species (ROS) by neutrophils, resulting in hyper-inflammation and recurring life-threatening infections. Mutations causing X-CGD span the entire 13 exons and intronic splice sites of the >30-kb *CYBB* gene, each resulting in a loss of gp91phox protein expression. We previously described the efficient targeted correction of a specific single mutation in *CYBB* exon 7 of patient CD34⁺ hematopoietic stem cells upon electroporation with an exon 7-specific CRISPR/Cas9 RNA and a single-stranded oligo DNA donor for homology-directed repair, which restored gp91phox protein expression and ROS production following multi-lineage engraftment in NSG mice. To extend this targeted gene correction approach to a broader group of patient mutations, we now report the use of exon 7-specific CRISPR for targeted insertion of an AAV6 donor construct containing a normal cDNA for *CYBB* exons 7 through 13 (CYBB E7-13), which encompasses approximately 60% of known X-CGD patient mutations. Electroporation of patient CD34⁺ cells with CRISPR/Cas9 RNA and CYBB E7-13 AAV resulted in efficient targeted CYBB E7-13 insertion as shown by the restoration of gp91phox protein expression in ~50% of neutrophils differentiated from the electroporated CD34⁺ cells *in vitro*, relative to control neutrophils differentiated from normal CD34⁺ cells. To further expand the *CYBB* cDNA correction strategy to encompass >90% of X-CGD patient mutations, we extended the approach to achieve targeted insertion of an AAV6 donor containing *CYBB* cDNA for exons 2 through 13 (CYBB E2-13) at the *CYBB* exon 2 locus using an exon 2-specific CRISPR/Cas9; exon 2 was chosen for targeting in this approach based on our earlier findings that correction at *CYBB* exon 1 is problematic because elements in intron 1 may be essential for gp91phox expression from the *CYBB* promoter. Electroporation of X-CGD patient CD34⁺ cells with the exon 2 CRISPR/Cas9 RNP resulted in ~60% indel activity, and concurrent transduction of RNP-electroporated cells with CYBB E2-13 AAV donor resulted in restoration of gp91phox expression and ROS activity in 23-29% (n=3) of mature neutrophils differentiated from X-CGD CD34⁺ cells relative to normal controls; on a per-cell basis, corrected neutrophils exhibited 93-95% of normal levels of ROS production based on mean fluorescence intensity by dihydrorhodamine flow cytometry assay. Together, our data demonstrate the highly efficient targeted integration of *CYBB* cDNAs at the endogenous locus in X-CGD CD34⁺ cells to achieve physiological levels of protein expression and ROS function that will treat the majority of X-CGD mutations. *In vivo* studies are currently underway to assess the retention of hematopoietic stem cell function and engraftment potential following targeted correction.

499. Efficient Editing of the CD40LG Locus in Human Hematopoietic Stem Cells

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X-linked Hyper IgM Syndrome (X-HIGM) is caused by mutations in the *CD40LG* gene. CD40L is primarily expressed on the surface of activated T cells, but can also be found on activated B cells, platelets, macrophages, and NK cells. CD40L:CD40 co-stimulatory signals are essential for triggering class-switch recombination (CSR) and somatic hyper-mutation in germinal center B cells. Patients suffering from X-HIGM present with elevated levels of IgM, but very low levels of IgA, IgE, and IgG, and suffer from recurrent viral and bacterial infections. At present, the only curative treatment for X-HIGM is allogeneic bone marrow transplant; however, many patients lack a suitable donor. Studies of female carriers of X-HIGM demonstrate that a relatively small proportion of CD40L-expressing T cells may be sufficient to reconstitute immune function, suggesting that genetic correction of as few as ~5-10% of autologous stem cells may provide curative therapy. Notably, gene therapy using γ-retrovirus delivery of CD40L cDNA led to T cell lymphoproliferative disorder in *Cd40l-/-* mice, indicating that endogenous transcriptional regulation will be important for successful therapy. Here, we report a homology directed repair (HDR)-based gene editing approach, where the CD40L cDNA is under control of the endogenous promoter. Previously, we described a gene editing strategy using TALENs and AAV6 to introduce a CD40L cDNA downstream of the endogenous CD40LG promoter in primary human T cells. Gene-edited T cells expressed CD40L at levels and kinetics that matched those of the endogenous protein in non-edited cells and allowed X-HIGM T cells to signal B cell CSR. Using this strategy to edit human CD34+ peripheral blood stem cells (PBSC) obtained from GM-CSF-mobilized donors, we first compared editing using *CD40LG*-specific TALEN vs. Cas9 RNP. We initially used AAV6 donor template to deliver an MND-GFP reporter

construct into the target site as the *CD40LG* promoter is not active in early hematopoietic cells. We found 50% higher rates of gene-editing and slightly improved cell viability using Cas9 RNPs (average of 30% HDR; N=9, 3 donors) vs. the TALENs (average of 20% HDR; N=11, 4 donors). We then used Cas9 RNPs and an AAV6 donor template to deliver *CD40L cDNA* under control of the endogenous promoter and found up to 30% of cells expressing our repair template at the genome level by ddPCR. Edited cells expressing GFP were transplanted into NOD-*scid*-IL2Rg^{NULL} mice to assess editing of long-term hematopoietic stem cells. Using optimized editing conditions with RNP and AAV6 donor, 12-16 weeks after transplantation, an average of ~4% (range 1-10%; 3 exp; 1 donor) of human cells in the bone marrow were GFP+, indicating the ability of edited cells to engraft. In all recipients, edited cells were present in myeloid, granulocyte, and B cell populations at ratios that were similar to non-edited human CD34+ cells, suggesting that the differentiation capacity of edited cells is not compromised. Additionally, we tested the addition of small molecules that have been reported to enhance the self-renewal of LT-HSCs during the ex- vivo culturing and gene editing steps of our protocol. While none of the small molecules tested significantly increased the engraftment of edited cells, the overall proportion of engrafted cells was increased using a subset of these small molecules. Overall, our findings demonstrate efficient editing of the *CD40LG* locus in human hematopoietic stem cells at levels predicted to provide clinical benefit.

500. Transplantation of Fetal Recipients with Placental Cells Engineered to Express FVIII Leads to Corrective Plasma Levels of FVIII after Birth

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Prenatal transplantation (PNTx) is a clinically viable procedure that poses minimal risk to both the fetus and the mother. Hemophilia A (HA) is an ideal disease to treat by PNTx, since 75% of HA patients have a family history of HA, and prenatal diagnosis is feasible and readily available. Successful PNTx for HA could promise the birth of a healthy infant requiring no further treatment. Even if not curative, an increase in FVIII levels, provided by the transplanted FVIII-expressing cells, could convert a severe, life-threatening bleeding disorder to a mild phenotype, reducing the need for postnatal treatments and inducing immune tolerance to FVIII. Here, using a large animal model of PNTx, we tested an off-the-shelf therapy that could be provided to HA patients prior to birth. We first demonstrated that human placental cells (PLCs) (passage 3-15) expressed vWF, constitutively produced and secreted functional FVIII at 0.3±0.2 IU/10^6 cells/24h, and, following lentiviral vector (LV) transduction, these cells increased their production and secretion of FVIII by 28-30 fold. Characterization of PLCs at passage 3 after transduction with a LV encoding a bioengineered, codon-optimized FVIII transgene, designated mcoET3, under the control of the EF1α promoter, demonstrated that PLCs stably secreted 8.44IU FVIII/10^6 cells/24h with a vector copy number of 0.35±0.05 per diploid genome equivalent. To test the therapeutic potential of the PNTx-based treatment for HA, mcoET3-LV transduced human PLCs were transplanted into sheep fetuses (n=11) at doses of 10^7-10^8 cells/kg fetal weight, at 65-71 gestation days (gd; term 145 gd), the equivalent of 16-17 gestational weeks in humans, using a clinically-employed ultrasound-guided injection procedure. Thus far, 6 of the transplanted animals have been born, and have been evaluated for 5 to 11 months after transplant, 3 animals were lost to non-procedure-related morbidities, and 2 are yet to be born. Evaluation at 4 and 5 month post-transplant (n=5) showed increases in plasma FVIII activity levels, as measured by aPTT, of 36-237% and 30-265%, respectively, when compared to control non-transplanted animals. Of particular note is that comparison of plasma analysis between 4 to 5 months post-transplant demonstrated that plasma FVIII levels were maintained in all 5 animals, despite the more than tripling in plasma volume that occurred during this infancy period of rapid growth. Follow-up of animals at 7.5 months (n=3) and 11 months (n=2) post-transplant demonstrated persistence of elevated FVIII activity in each animal. Specifically, all animals continue to have plasma FVIII activity levels that exceed that of control non-transplanted animals by over 30%. In conclusion, we have shown that human PLC are an ideal off-the-shelf cellular vehicle for delivering a FVIII transgene, and that PNTx with PLC engineered to express an expression/secretion-optimized variant of FVIII (mcoET3) results in curative plasma levels of FVIII following birth, in a large preclinical animal model.

501. Gene Therapy of Murine Thalassemia by *In Vivo* Transduction of Mobilized Hematopoietic Stem Cells with an Integrating Hybrid Adenovirus Vector System

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Currently used *ex vivo* hematopoietic stem cell (HSC) gene therapy is a complex and expensive procedure requiring extensive HSC manipulation and transplantation expertise. Moreover, the intense myeloablative conditioning required to reach clinically relevant HSC engraftment levels, increases toxicity and prolongs hospitalization. We developed a minimally invasive and readily translatable approach for *in vivo* HSC gene delivery without leukapheresis, myeloablation, and HSC transplantation. It involves injections of G-CSF/AMD3100 to mobilize

HSCs from the bone marrow into the peripheral blood stream and the intravenous injection of an integrating, helper-dependent adenovirus (HDAd5/35++) vector system. HDAD5/35++ vectors target CD46, a receptor that is expressed on primitive HSCs. Transgene integration is achieved (in a random pattern) using a hyperactive Sleeping Beauty transposase (SB100x). We demonstrated in adequate mouse models, using GFP as a transgene, that primitive HSCs transduced in the periphery home back to the bone marrow where they persist and stably express GFP long-term. We here pursued this approach for the treatment of hemoglobinopathies by incorporating the human γ-globin gene under the control of the β-globin LCR, containing HS1 to HS4 and the β-globin promoter into HDAD5/35++ vectors. Given the high level of transgene marking required in differentiated peripheral blood cells in order to phenotypically correct thalassemia, we also inserted the $mgmt^{P140K}$ gene into the vector as a means to increase the frequency of γ-globin expressing red blood cells by *in vivo* selection with low-dose O^6BG/BCNU treatment post mobilization/transduction. We tested our approach in a mouse model for β-thalassemia intermedia (Hbb^{th-3}/$hCD46^{++}$ mice). Mobilization yielded high numbers of circulating Lin^- $Sca\text{-}1^+ckit^+$cells (270±27 LSK/μl of blood). At week 8 post transduction, yet in the absence of *in vivo* selection, and probably as a result of a selective advantage provided by the thalassemic background to the gene-corrected cells, Hbb^{th-3}/$hCD46^{++}$ mice expressed HbF in 33.6±16.03% of circulating erythrocytes. Due to a significant drop in HbF expression in blood (11.9±3.0%) at week 16, a 3-dose O^6BG/BCNU treatment was initiated in a cohort of mice, thus recovering HbF expression (62±7.2%) of circulating erythrocytes. Post O^6BG/BCNU treatment, significant phenotypic improvement was observed (improved red cell morphology in blood smears, increased Ht/Hb and red cell indices, decreased reticulocytes). Secondary, lethally irradiated C57Bl/6 recipients transplanted with bone marrow Lin^- cells from a cohort of Hbb^{th-3}/$hCD46^{++}$ mice not subjected to *in vivo* selection, provided high levels of engraftment and γ-globin expressing red cells (58.3±5.1%) six weeks post-transplant. Quantitative measurements of γ-globin protein and mRNA as well as integration site analysis are in process. Overall, we present data on a simplified HSC gene therapy of thalassemia, which can serve as a cost-efficient and "portable" approach, especially in developing countries, where only minimally complex strategies for hemoglobinopathies could be adopted.

502. Simultaneous Disruption of the Erythroid Enhancer of BCL11a and of a BCL11a-Binding Site in the Beta Globin Locus Synergistically Increases Endogenous Fetal Hemoglobin Expression

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Despite the significant advances on developing novel therapies for hemoglobinopathies, a universal and robust therapeutic approach able to achieve an event-free result in all β0/β0 thalassemic and sickle cell patients is not yet available. Thalassemia or sickle cell patients with increased Fetal hemoglobin (HbF) have an ameliorated clinical picture up to transfusion independency. Inactivation via genome editing of the γ-globin suppressor BCL11a or introduction of Hereditary Persistence of Fetal Hemoglobin (HPFH) mutations in the hemoglobin gamma gene (HBG) promoter has been shown to significantly increase the endogenous HbF expression. In order to achieve higher efficiency in HbF reactivation, we simultaneously introduced mutations in the BCL11a-erythroid enhancer and in the recently identified BCL11a binding site on the HBG promoter. Transduction of human erythroid progenitors with a dual CRISPR/Cas9 helper dependent-adenovirus vector (HD-Ad5/35++), permitting simultaneous targeting of the two different DNA loci, greatly enhanced expression of the endogenous fetal globin, superior to the increase observed by either single mutation. Specifically, simultaneous disruption of both loci in human erythroid progenitors increased the overall HbF+ cells from less than 2% in the control to more than 60% (single knock outs:18-50% HbF+ cells), and HbF expression from less than 1% to more than 20% of adult globin (single knock outs: 5-10%). Evaluation of single clones after editing, revealed a significantly higher fetal globin expression in the double knock out clones than the single ones implying a possible synergistic effect of the two mutations. In addition to the in vitro studies, we showed that CD34+ cells edited in both loci, achieve a similar level of human chimerism in xenorecipients to the single locus edited cells, suggesting that the introduction of two mutations does not compromise the engraftment potential. Furthermore, the higher HbF levels appear to be retained in vivo. This strategy has the potential to induce higher levels of HbF reactivation with a clinical benefit in patients with beta globin disorders.

503. AAV-Delivered eCD4-Ig Protects Rhesus Macaques from High Dose SIVmac239 Challenges

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Despite the absence of an effective HIV-1 vaccine, numerous broadly neutralizing antibodies (bNAbs) have been isolated that target different epitopes on the HIV-1 envelope glycoprotein (Env). Some bNAbs potently neutralize >90% of HIV-1 isolates tested, yet no bNAb described is effective against all HIV-1 isolates. We have reported that eCD4-Ig, an HIV-1 entry inhibitor that fuses a CCR5-mimetic peptide to the C-terminus of CD4-Ig, is broader than any bNAb described to date. eCD4-Ig neutralizes 100% of HIV-1, HIV-2, and SIV isolates at concentrations comparable to the best bNAbs. We previously demonstrated that AAV vectors encoding a rhesus macaque version of eCD4-Ig (rh-eCD4-Ig) could express rh-eCD4-Ig for over a year. The concentrations of rh-eCD4-Ig protected the macaques from multiple escalating challenges of SHIV-AD8. In this study, we examined whether eCD4-Ig could protect macaques from SIVmac239 challenges. Four macaques were intramuscularly inoculated with AAV1 vectors to express functional rh-eCD4-Ig. Macaques expressed peak concentrations of rh-eCD4-Ig that ranged from 13-44 μg/mL and had set point concentrations ranging from 3-18 μg/mL. All macaques had an anti-drug antibody response (ADA) against the CCR5-mimetic peptide of the inhibitor. However, the ADA response decreased to background levels in all four macaques by 32 weeks post inoculation. SIVmac239 challenges were started at 22 weeks post inoculation and all eight control macaques became infected after the second challenge

at a dose of 40 pg p27. We observed significant protection (p=0.002, Mantel-Cox test) in the four macaques expressing rh-eCD4-Ig. These macaques were infected on the third, fifth, sixth, and seventh challenges at doses of 80, 320, 640, and 1280 pg p27, respectively. Macaques expressing rh-eCD4-Ig had significantly lower peak and set point viremia compared to the control group. Furthermore, we observed mutations in the CD4-binding site of the SIVmac239 Env at 22-38 weeks post infection. One mutation, a G384 deletion, conferred moderate resistance to rh-eCD4-Ig based on neutralization assays. However, a second mutation G382R did not mediate resistance to rh-eCD4-Ig, rather, it facilitated greater entry and infection in cells expressing rhesus macaque CD4. These studies demonstrate that AAV-delivered eCD4-Ig is the first HIV vaccine of any kind to show complete and significant protection against both a SHIV isolate and SIVmac239. We conclude that AAV-delivered eCD4-Ig to be a viable alternative to conventional HIV-1 vaccine strategies.

504. Intravenous Delivery of Lentiviral Vectors to Treat Hemophilia A in a HSC Mobilization Mouse Model

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We utilized a new *in vivo* approach to genetically modify hematopoietic stem cells (HSCs) by lentiviral vector (LVs) for the treatment of Hemophilia A. This approach can avoid several potential risks posed by ex vivo gene therapy. We first mobilized primitive HSCs from bone marrow (BM) to peripheral blood by administering a combination of G-CSF and AMD3100 in mice (mobilization group), followed by intravenous injection (IV) of lentiviral vectors (LVs). The control non-mobilization group was treated with LVs only. We investigated gene delivery efficiency by using four LVs encoding the GFP or FVIII gene, either driven by ubiquitous promoters or a megakaryocytes specific glycoprotein-1 bα promoter (MND-GFP-LV, G-GFP-LV, EF-FVIII-LV, G-FVIII-LV). Following IV delivery of MND-GFP-LV, GFP expression in HSCs can be detected by flow cytometer at 4 weeks (13.35±2.5% of total HSCs) and 10 weeks (6.71±3.9%), respectively. In the non-mobilization group the percentage of HSCs transduced is similar to that of the mobilization group, indicating that MND-GFP-LV can readily transduce HSCs in either blood or BM via IV injection. However, mean fluorescence intensity (MFI) of the GFP^+ signal in the mobilization group is 5 times higher than that in the non-mobilization group. These results confirmed that IV delivery of LVs can efficiently transduce HSCs mobilized to the peripheral blood and the transduced HSCs then home back to BM from the circulation. In addition, GFP expression directed by Gp1bα promoter was observed in ~0.2% platelets of G-GFP-LV treated mobilization group of mice at 10 weeks post LV delivery, whereas no GFP expression was detectable in non-mobilization group. These results indicate that transduction efficiency of G-GFP-LVs is significantly enhanced by HSC mobilization to allow increased GFP expression in megakaryocytes and storage in platelets. Furthermore, to demonstrate the therapeutic benefits of this novel strategy, we treated HemA mice by IV injection of the EF-hFVIII-LVs. In both mobilization group and non-mobilization group, the circulatory FVIII activity in plasma was detected by APTT assay, but FVIII expression decreased to undetectable levels at day 42 post LVs injection, which correlated with the appearance of anti-FVIII inhibitory antibodies. Thus, FVIII expression driven by ubiquitous promoters tends to induce FVIII naturalizing antibodies after 42 days post injection. Delivery of G-FVIII-LVs to express FVIII in platelets has the potential to escape immune response against FVIII. Following IV delivery of G-FVIII-LVs, we found that there were 2-10% platelets containing FVIII in the mobilization group during 3 months experimental period, whereas background levels were observed in the platelets of non-mobilization group. In carotid artery injury experiments after 4 months treatment, the blood flow rate of the G-FVIII-LV treated mice decreased significantly compared with the mice without LVs treatment. Overall, we have demonstrated that a single IV injection of LVs into HSC mobilized mice can significantly increase the number of transduced HSCs in bone marrow. Most significantly, IV delivery of G-FVIII-LV achieved long-term FVIII expression in platelets and partial correction of hemophilia A phenotype. This *in vivo* approach has high potential for an effective treatment of FVIII deficiency in patients.

505. Non-Viral Engineered CAR-T Cells for Safe and Specific Stem Cell Transplant Conditioning

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Each year in the U.S. over 5,000 patients with hematological disorders are treated with myeloablative conditioning therapy prior to reconstitution by transplanted hematopoietic stem cells (HSCs). These conditioning regimens typically consist of high doses of genotoxic radiation or busulfan, which can lead to life-threatening post-transplant complications. This has prompted the investigation of more targeted and less hazardous approaches to specifically deplete endogenous hematopoietic cells in the bone marrow (BM). To date, antibodies broadly directed against HSC antigens, such as c-kit and CD45, have been considered for transplant conditioning. However, this approach is limited by the slow rate of antibody clearance, which delays time to transplant, and wide biodistribution, which limits efficacy and increases the possibility of toxicity towards non-HSC antigen-bearing cells. Alternatively, short-lived, chimeric antigen receptor (CAR)-T cells with bone marrow-homing capability may provide more effective, selective, and safer depletion of resident HSCs. Among the CAR-T production protocols currently in development, delivery of the CAR transgene via the non-viral *piggyBac*™ (PB) transposon method has several advantages: (1) lower manufacturing costs, (2) larger cargo capacity that allows introduction of multiple genes, and (3) a predominantly stem cell memory (T_{SCM}) phenotype for bone marrow homing ability and enhanced *in vivo* potency. Here, we constructed PB vectors encoding CARs targeting either human c-kit (CD117) or prominin-1 (CD133), markers known to be antigenically expressed on HSCs. In addition to the CAR, the transposon also encodes a selection

gene, for generation of entirely pure product, and an inducible safety switch that will allow rapid clearance of the reactive CAR-T cell product prior to donor HSC transplant. A panel of CAR candidates were first evaluated *in vitro* for their ability to specifically deplete human myeloid leukemia cells (TF-1a) expressing c-kit and CD133. Two-day co-culture of select anti-c-kit or anti-CD133 CAR-T cells with primary human or Rhesus macaque BM cells resulted in greater than 80% depletion of functional hematopoietic subsets as determined by assays quantifying the frequency of colony forming cells (CFUs) and the more primitive cobblestone area forming cells (CAFCs). The PB CAR-T manufacturing process yielded CAR-T cells with more than 60% T_{SCM} content, as determined by CD62L, CCR7, and CD45RA expression. The CAR-T cells also expressed high levels of CXCR4, a key chemokine receptor involved in selective BM trafficking. Studies evaluating the *in vivo* efficacy of lead anti-HSC CAR-T candidates are underway in immunodeficient NSG mice engrafted with either a human acute myeloid leukemia cell line or primary human CD34+ hematopoietic stem and progenitor cells. Our findings provide proof-of-concept that controllable and non-genotoxic CAR-T cells directed against c-kit or CD133 bearing hematopoietic cells can be used to safely and specifically eliminate HSCs in order to allow engraftment of allogeneic or gene-corrected stem cells. This may ultimately lead to safer conditioning protocols that greatly increase the number of patients eligible for transplant and significantly reduce the damaging side-effects associated with current radiation or chemotherapy treatment.

506. Abstract Withdrawn

507. Abstract Withdrawn

508. Efficient Targeted Integration in Human T Cells with CRISPR-Cas9 for the Treatment of X-Linked Hyper-IgM Syndrome

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X-linked Hyper-IgM Syndrome (X-HIGM) is a rare primary immunodeficiency characterized by recurrent infections and reduced life expectancy. X-HIGM is caused by mutations in the *CD40LG* gene which leads to loss of expression of CD40 ligand (CD40L), defective B-cell class switching, increased serum IgM levels, and an inability to mount an adaptive immune response. Tight regulation of CD40L is critical as it has been shown that lentiviral-mediated constitutive expression of CD40L is causative of lymphoproliferative disease in mice. Therefore, *CD40LG* has not been considered a good candidate for classical gene therapy since it's essential to keep CD40L under its endogenous promoter. The mutations in *CD40LG* are diverse in type and are scattered throughout the exons and introns of this gene. We will present our therapeutic strategy for cDNA replacement by targeted integration in $CD4^+$ T cells at *CD40LG* endogenous locus. We screened *CD40LG* locus for the best gRNA, based on efficacy of cutting and then selected gRNAs that achieve > 85% cutting in primary human T cells. We also evaluated specificity utilizing *ex vivo* and *in vitro* unbiased methods. We identified 4 lead gRNAs (*S. pyogenes and S. aureus*) with high efficiency and specificity profiles and developed AAV6 donors that restore functional CD40L expression. We observed differences in targeted integration among the Cas9 variants and locations within the *CD40LG* locus that were evaluated. Finally, we achieved >40% targeted integration in human $CD4^+$ T cells leading to expression of the CD40L protein at a therapeutically relevant level. Our successful targeted integration at the *CD40LG* locus in $CD4^+$ human T cells provides the foundation to develop other gene correction and replacement strategies for diseases for which endogenous control of gene expression may be therapeutically beneficial, such as Sickle cell disease and beta-thalassemia.

509. Genome Editing for IL-10 Deficiency in Purified Hematopoietic Stem Cells

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Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine, produced by various immune cells, including B- and T-cells, macrophages, monocytes, dendritic cells and mast cells, as well as non-immune cells such as keratinocytes and epithelial cells. IL-10 plays a key role in preventing excessive inflammatory responses and it is essential for immune tolerance and gut homeostasis. Homozygous loss of function mutations in *IL-10* cause severe infantile inflammatory bowel disease. These patients are typically resistant to conventional immunosuppressive therapies whereas allogeneic hematopoietic stem cell transplantation (allo-HSCT) leads to sustained remission and it is currently considered the only curative treatment. However, limited donor availability and morbidity and mortality due to allo-HSCT complications prevent its wide use in these primary immune regulatory disorders. Hence, an approach based on gene-correction of hematopoietic stem and progenitor cells (HSPCs) derived from patients will allow us to use an autologous HSCT. CRISPR/Cas9 system is a powerful method which allows precise genetic manipulations and can represent a curative strategy several human genetic diseases. The severe immune dysregulation in IL-10 deficient patients and the poor long-term prognosis, make this genetic disease an ideal candidate for genome engineering. We used CRISPR/Cas9 technology to design a strategy for *IL-10* gene correction and also to identify new factors regulating IL-10 expression. We delivered CRISPR/Cas9 as a ribonucleoprotein (RNP) complex in HSPCs via electroporation, together with chemically modified guide RNAs (gRNAs), targeting *IL-10* 5'UTR and exon 1 (Fig1). We tested the activity of seven gRNAs measuring INDELs frequencies. The targeted genomic sites were amplified by PCR reaction on the extracted gDNA samples from transduced cells and to assay INDELs levels, we used Tracking of INDELs by Decomposition (TIDE) software. Results from TIDE analysis are depicted in Fig.2a. IL-10-3, -5 and -6 gRNAs were selected for further experiments, because of the high INDELs efficiency (IL-10-5=92%; IL-10-6=72%) or proximity to start codon (IL-10-3) (Fig.2b). We designed DNA donor templates to generate both KO and gene editing. We are currently determining the frequencies of homologous recombination (HR) mediated by selected

gRNAs and off-target events. The function of edited HSPCs will be tested *in vitro* and *in vivo* humanized mouse models. Overall, using CRISPR/Cas9 technology, we will develop an efficient method for safe IL-10 gene correction that can be used as a definitive and curative treatment of IL-10 deficiency. In addition, this approach will allow us to investigate mechanisms underlying transcriptional regulation of the IL-10 gene.

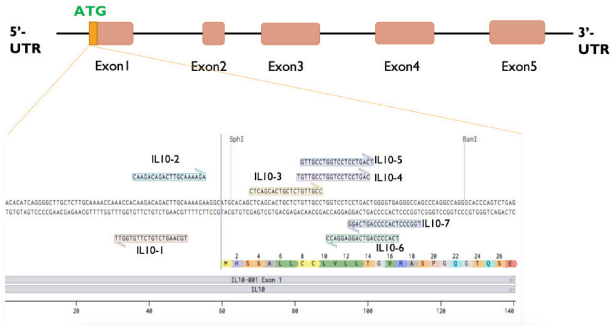

Fig1: gRNAs targeting IL-10 gene.

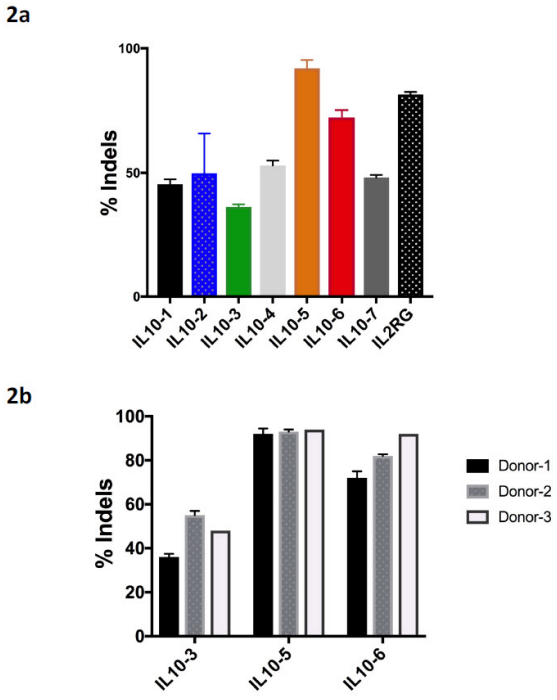

Fig2: INDELs frequency. a) HSPCs were electroporated with RNP CRISPR system, and INDELs were analysed via TIDE ($n=1$, CD34+ mobilized peripheral blood). Bars represent SD from duplicates. IL2RG is positive control. **b)** HSPCs treated as above in 2 additional donors.

510. Enhancing Immune Regulation to Promote Tolerance to Factor VIII in Gene Therapy for Hemophilia A

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A recent clinical trial showed AAV gene therapy to be effective in restoring clotting factor VIII (FVIII) levels to normal in severe hemophilia A patients. However, all patients, even after corticosteroid treatment, showed significant elevation in serum alanine aminotransferase (ALT), indicating liver damage. Furthermore, patients with a history of antibody ("inhibitor") formation against FVIII were excluded from the study. This serious complication occurs in 20-30% of severe hemophilia A patients in response to FVIII replacement therapy. While optimal hepatic AAV gene transfer typically induces tolerance to the transgene product, this is not always observed for FVIII. We previously showed that a combination of antigen, the cytokine Flt3L, and the mTOR inhibitor rapamycin can induce tolerance to proteins via deletion of effector T cells and induction of Treg, which is enhanced by selective expansion of plasmacytoid dendritic cells (pDC). Here, we sought to test whether such a regimen can promote tolerance to FVIII expressed from an AAV vector. In the first of two experiments, we gave an intravenous cocktail of Flt3L, rapamycin, and FVIII antigen 3x/week for 4 weeks to hemophilia A mice (BALB/c F8e16$^{-/-}$, n=8). Three weeks into the regimen, we administered AAV8 expressing codon optimized B-domain deleted human FVIII (AAV8-BDD-hFVIII) intravenously at a dose of 1x10^{11} vg, and continued tolerization for one more week. Non-tolerized control mice received vector only (n=7). Both experimental and control groups had FVIII activity at two weeks following AAV treatment. By four weeks, the control group had formed inhibitors against FVIII and lost coagulation activity. Although by eight weeks 6 mice in the experimental group had formed inhibitors, their FVIII activity stayed above control group levels. Moreover, 2 experimental mice had minimal loss of FVIII activity without evidence of inhibitor formation. In the ongoing second experiment, treatment groups included either Flt3L and rapamycin, Flt3L and increased rapamycin, or rapamycin alone (total of 23 hemophilia A mice). Here, AAV8-BDD-hFVIII was given after one week of the tolerization regimen, which was subsequently continued for a total of 10 weeks. Control mice again received AAV-FVIII only. FVIII activity is being measured by chromogenic assay, FVIII inhibitors (BU/ml) by modified Nijmegen-Bethesda assay, and anti-FVIII IgG titers determined by ELISA. Frequencies of DC subsets and Treg are being determined by flow cytometry. In conclusion, results from this strain of mice that fails to achieve immune tolerance to FVIII from hepatic AAV gene transfer alone suggest that an immune modulatory regimen that enhances induction of FoxP3^{+} Treg improves the outcome of gene therapy. Optimization of the regimen should further enhance immune regulation to more completely prevent inhibitor formation.

511. Lentiviral Vector Based Gene Therapy for Major Haemoglobin Disorders

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β-thalassemia major and sickle cell disease are the most common major haemoglobin disorders. Even though allogeneic hematopoietic stem cell transplantation (HSCT) has been available for >35 years for these patients, <10,000 alloSCTs have been performed during this period. (*Haematologica*, 102:2-11, 2017). Several groups have reported successful lentiviral vector based gene therapy for these disorders in recent years. We have evaluated three lentiviral vectors (CMCEUv1, CMCEUv3 and CMCEUv4) for corrective gene transfer of the β-globin gene. These are 3rd-generation self-inactivating vectors with a LTR sequence. For erythroid-specific expression a 3.1 kb fragment of locus control region of beta globin cluster containing the DNase hypersensitive sites, HS4, HS3, and HS2, were included. While CMCEUv1 has a 180bp β-promoter, CMCEUv3 has the same but with a co-transcriptional cleavage (CoTC) sequence and CMCEUv4 has a 266bp β-promoter with the same CoTC sequence. The T87Q mutation was also introduced into the beta globin coding sequence to enhance its anti-sickling property and induce a different electrophoretic mobility. These vectors were systematically evaluated in two preclinical models: a two-phase *ex-vivo* human erythropoiesis model and the Townes mouse model of sickle cell disease (hα/hα: β^S/β^S). The former is a two-phase liquid culture system involving expansion and differentiation of HSCs. In brief, CD34+ cells were cultured in expansion media in presence of SCF, Flt3, IL3, IL6 in phase 1 followed by a phase 2 culture with SCF, Epo, and IL3. The erythroid differentiation is then assessed by expression of Glycophorin A and transferrin. cDNA from these cells is amplified for the β-globin exon2 using FAM labelled primers and subjected to DraIII restriction digestion. With this, we could demonstrate that about 16% of the β -globin transcripts derived from the transduced HSCs were from the transgene (Figure 1) The transduced HSCs had vector copy numbers (VCNs) of 1.1-3. In the *in vivo* mouse model of sickle cell disease, transduced $Sca1^+$ cells harvested from SCD mice were transplanted into lethally irradiated congenic C57BL/6 mice and were observed for haemoglobin expression, complete blood counts and oxygen saturation. A gradual increase in the transgene haemoglobin was observed up to 17 weeks (Figure 2) CMCEUv1 and CMCEUv4 showed better transgene expression than CMCEUv3. We also demonstrated an improvement in the pO_2 levels from 32mM of Hg to 24 mm Hg in transplanted mice as well as a reduction in sickling index in the transplanted mice, and near normalization of the white blood counts. These data show that these vectors are effective in transferring the β-globin transgene and expressing significant quantities of haemoglobin. The best among these would need to be tested in human HSCs transplanted in a suitable pre-clinical model. If successful, that could pave the way towards a clinical trial in the Asia Pacific regions, where there is a very high prevalence of these disorders.

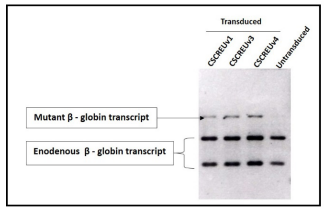

Fig 1. DraIII enzyme digestion of mutant and endogenous β – globin transcripts.

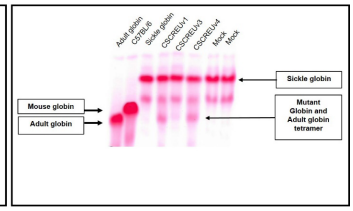

Fig 2. Cellulose acetate electrophoresis showing mutant globin expression from transplanted mouse samples after week 17.

512. Pairs of Guide RNAs Mediate Precise Deletions on the *PKLR* Gene via Non Homologous End Joining Generating a Human Hematopoietic Progenitor Model of Pyruvate Kinase Deficiency

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Pyruvate kinase deficiency (PKD) is a rare disease and the main cause of hemolytic non-spherocityc anemia being fatal in some cases during early childhood. This autosomal recessive disorder is produced by mutations in PKLR gene. Our group developed a gene therapy protocol based on the ex vivo correction of hematopoietic progenitors using a lentiviral vector (LV) which carries a codon-optimized version of the PKLR cDNA (coRPK). The vector has been successfully tested in mice and it has been designated as Orphan Drug for the treatment of PKD by the EMA and FDA agencies. To test the developed tools in human cells, hematopoietic progenitors are required. However, bone marrow aspirations are not part of the follow up of the patients meaning that PKD hematopoietic progenitors are not samples easy to obtain. To overcome this limitation, we generated PKD-like hematopoietic progenitors modifying healthy CD34+ progenitors using a CRISPR/Cas9-2A-ZsGreen plasmid construct. Edited cells were selected by sorting based on ZsGreen protein, achieving populations near 100% modified by means of non-homologous end joining (NHEJ). Interestingly, electroporation of guide pairs allowed us to control NHEJ events, producing the precise deletion between guides. Six guide RNA pairs were tested, deleting 8, 30, 60, 120, 240 and 500 bp. Precise deletion was the predominant event in all pairs tested except the 8 bp deletion. Thirty bp deletion was used to verify the generation of PKD cells. Neither PKD-like nor PKD patient cells seems to be impaired to reach the same maturation along erythroid maturation stage as control cells. Moreover, Pyruvate kinase activity was impaired on PKD-like cells in a similar extent as PKD patient´s cells after *in vitro* erythroid differentiation. These results demonstrate that the generation of PKD-like cells via CRISPR/Cas9 edition is feasible and generate PKD deficient cells suitable to be used for testing gene therapy strategies for the treatment of PKD.

513. Fetal Hemoglobin De-Repression Following CRISPR-Cas9-Mediated Targeting of the γ-Globin Promoters

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β-hemoglobinopathies (β-thalassemia and Sickle Cell Disease, SCD), the most prevalent genetic disorders worldwide, are caused by mutations affecting quantitatively or qualitatively the production of the adult hemoglobin (Hb) β-globin chain. In β-thalassemia, the reduced production of β-chains causes α-globin precipitation, ineffective erythropoiesis and insufficiently hemoglobinized red blood cells (RBC). In SCD, the $\beta 6^{Glu \rightarrow Val}$ substitution leads to Hb polymerization and RBC sickling, which is responsible for vaso-occlusive crises, hemolytic anemia and organ damage. The clinical severity of β-hemoglobinopathies is alleviated by the co-inheritance of genetic mutations causing a sustained fetal γ-globin chain production at adult age, a condition termed hereditary persistence of fetal hemoglobin (HPFH). Elevated fetal γ-globin levels reduces globin chain imbalance in β-thalassemias and exert a potent anti-sickling effect in SCD. Naturally occurring HPFH point mutations identified in the promoters of the two γ-globin genes, *HBG1* and *HBG2*, cluster at several loci, and are thought either to generate *de novo* DNA motifs recognized by transcription activators or disrupt binding sites for transcriptional repressors. Here, we have compared the extent of fetal hemoglobin (HbF) de-repression following CRISPR/Cas9-mediated targeting of different regions of the *HBG1* and *HBG2* promoters in an adult erythroid cell line (HUDEP-2). We achieved a potent and pancellular HbF re-activation upon disruption of a putative binding site for γ-globin repressors located in both *HBG1* and *HBG2* genes. Ongoing studies aim at validating these findings in RBC derived from genome edited healthy donor and patient hematopoietic stem/progenitor cells. Overall, this study identified a putative binding site for HbF repressors as a novel target for the treatment of β-hemoglobinopathies.

514. Development of Assays to Support Non-Clinical Studies for Human Factor IX

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Factor-IX is a serine protease in the coagulation cascade, and its deficiency is causally linked to Hemophilia B. It is synthesized as a zymogen and undergoes proteolytic processing and post translation Gla-domain modification, which are essential for generation of functional FIX. Herein, methods are described for quantification of human Factor-IX (hu-FIX), verification of its molecular weight and post-translational Gla modification. These methods can be used for analysis of hu-FIX spiked in murine, cynomolgus or rhesus plasma (non-human primate, NHP) matrices, each containing their respective endogenous FIX antigens. Multiple anti-FIX monoclonal antibodies were screened for their ability to recognize hu-FIX in normal human plasma, or recombinant hu-FIX spiked in murine and NHP plasma matrices. One monoclonal antibody (MAb01) was identified with anti-hu-FIX reactivity, and minimal cross reactivity to murine or NHP FIX antigen. A sandwich ELISA using this MAb01 was developed to accurately quantify hu-FIX antigen in ranges, which are relevant for normal or hemophilia B human subjects based on their corresponding FIX activity. Furthermore, similar performance was also observed in quantification of recombinant hu-FIX spiked in murine and NHP plasma matrices. An ELISA-activity assay was developed by using MAb01 to enrich hu-FIX in biological matrices, and then followed by FIX chromogenic activity assay as a readout. Chromogenic readout was only observed when hu-FIX was present in mouse or NHP plasma matrices, further confirming the specificity of MAb01. The hu-FIX antigen ELISA and ELISA-activity assay are complementary methods to quantify functional hu-FIX in biological matrices. To confirm the molecular weight and post translational Gla modification of hu-FIX, biological matrices were enriched for hu-FIX using MAb01, followed by denaturing PAGE and western blotting. Molecular mass of enriched Hu-FIX was identical to the recombinant FIX reference standard. In addition, western blotting with an anti-Gla domain antibody generated a readout with identical properties as the hu-FIX western blot indicating that this modification of functional FIX could be detected. In addition to MAb01, an anti-huFIX-Thr148 specific MAb02 was identified and was able to distinguish between Thr/Ala148 alleles, naturally occurring variants of hu-FIX. Thus, these newly developed assays for human Factor-IX could be useful to support non-clinical studies of therapeutic development candidates for Hemophilia B.

515. CRISPR Cas9-Mediated Genome Editing for the Modelling and Correction of Wiskott-Aldrich Syndrome

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Wiskott-Aldrich syndrome (WAS) is a congenital immune disorder characterized by thrombocytopenia, eczema and immunodeficiency. WAS is caused by mutations in the WAS gene, which leads to compromised expression of the Wiskott-Aldrich Syndrome Protein (WASP). WASP plays a key role in hematopoietic actin cytoskeleton reorganization, and the deregulation of this process is responsible for the pathophysiology of WAS. The gene therapy clinical trials with γ-retroviral vector and self-inactivating lentiviral vector have shown considerable improvement in immunological parameters. However, the γ-retroviral vector studies were complicated by the insertional mutagenesis resulting in leukemia. Here we describe a CRISPR/Cas9 mediated approach for the targeted insertion and expression of WAS gene. Our WAS transgene has an 800bp homologous sequences on both the arms of the transgene to the AAVS1 integration site in the PPP1R12C gene of the 19q 13.3 region. The transgene is regulated by 1.6kb WAS proximal promoter sequence. The co-transfection of CRISPR Cas9 Ribonucleoproteins (RNPs) and the transgene resulted

in the homology-directed targeted insertion of WAS transgene in AAVS1 genome safe harbour sites in 293 T cells. The genome editing conditions for adult hematopoietic stem cells and progenitors (HSPCs) were optimised. The purified HSPCs were pre-cultured with cytokines for 48 hours and nucleofected with Cas9 RNPs and WAS transgene. The gene targeting to AAVS1 locus was confirmed by T7 assay showing the InDels at the AAVS1 locus and the integration PCR assay which has a forward primer on the AAVS1 locus and the reverse primer on the WAS transgene. The gene edited cells were further analysed for the retention of cell surface receptors for primitive HSCs and the progenitors. The TPO containing cytokine cocktail has been optimised for generation of Megakaryocytes and platelets from the HSPCs to study the function of gene-edited HSPCs. In addition, We have also knocked out the WAS endogenous gene in K562 and Jurkat T cell lines by using a CRISPR/Cas 9 RNPs targeting an exon1 and intron 1 junction of WAS gene. The knockout cells were isolated by the HDR mediated integration and expression of GFP reporter from the endogenous promoter. The knockout cells will serve as the human cellular model of WAS mimicking megakaryocytes, platelets and T cell defects. Our on-going experiments on validating WAS gene editing constructs in the cellular models will be discussed.

Immunological Aspects of Vaccines

516. Rapid Protection against *Zaire Ebolavirus* (EBOV) Infection in Mice by Fully Human DNA Expression Cassette-Delivered Monoclonal Antibodies (DMAbs) Engineered from Clones Isolated from Ebola Virus Disease Survivors

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In the 2013-2016 Ebola virus disease (EVD) epidemic in West Africa, an anti-glycoprotein (GP) monoclonal antibody (mAb) cocktail, ZMapp, was associated with favorable recovery in human patients. Since then, several mAb clones with greater potency than the ZMapp clones have been isolated from human EVD survivors. We recently described the engineering of synthetic plasmid DNA expression cassette-delivered monoclonal antibodies (DMAbs) as a strategy for *in vivo* administration of potent mAb clones that frequently require high-dose administrations to maintain protective serum trough levels or have properties incompatible with bioprocess manufacturing. The DMAb platform facilitates direct *in vivo* transfection of muscle cells to produce and secrete into the blood mAb at biologically relevant levels. We engineered >30 human IgG1 anti-GP DMAbs from clones isolated from human EVD survivors and targeting different regions of the GP glycan cap, fusion loop, chalice base, HR2 region, or MPER region. Anti-GP DMAbs, administered by intramuscular injection followed by CELLECTRA adaptive constant current electroporation, were detectable in BALB/c mice for >120 days and share similar binding, neutralization, and epitope mapping properties to their protein IgG counterparts. BALB/c mice (n=10/group) were administered DMAb-11, DMAb-30, or DMAb-34 and monitored for DMAb expression in serum. Mice were then challenged on day 28 following administration with a highly lethal 1000LD50 dose of mouse-adapted Ebola virus (strain Mayinga). We observed 100% protection from both mortality and morbidity following DMAb administration. A new combination of DMAb-11, DMAb-30, and DMAb-34 was co-delivered to BALB/c mice (n=10/group) as a DMAb-cocktail designed to evade potential Ebola virus escape mutants. The DMAb-11-30-34 cocktail was 100% protective at lower doses than the individual DMAbs. In a separate experiment, we administered DMAb-11 to BALB/c mice day 8 before lethal challenge, observing 80% protection. This study supports the efficiency of DMAb delivery as an intervention for highly pathogenic hemorrhagic fever viruses and potential of a DMAb-cocktail approach. These studies demonstrate the importance of *in vivo* DMAb delivery as a novel approach for preventing deadly emerging infectious diseases including Ebola virus disease, and has implications for prevention and therapy of a range of infectious diseases as well as cancers.

517. Replicating Single-Cycle Adenovirus Vaccine against Ebola Virus

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Although the most recent Ebola virus (EBOV) epidemic is officially over, a future resurgence of EBOV is a concern demanding diligence. Only two investigational vaccines have been field-tested and there is currently no FDA-approved vaccine. Among promising gene-based vaccine vectors are adenoviruses (Ads). The vast majority of Ad vaccines are RD-Ad vectors, which do not amplify the antigen genes they carry, and upon infecting a cell, express only "1X" antigen per cell. One of the field-tested vaccines in the 2014 outbreak was the chimpanzee-derived replication-defective adenovirus (RD-Ad), ChAd3-EBOV. While RD-Ads elicit robust protection, they require increased particle delivery and their efficacy wanes when tested in humans. Alternatively, replication-competent Ads (RC-Ad) can replicate genes up to 10,000-fold to amplify antigen expression and immune responses. While RC-Ads are more potent, they run the risk of causing adenovirus disease due to uncontrolled viral replication. To circumvent these pitfalls, we recently described a "single cycle" adenovirus (SC-Ad) vector that amplifies antigen genes like RC-Ad, but avoids the risk of adenovirus infection. We have tested an SC-Ad6 vector expressing the glycoprotein from a 2014 Zaire EBOV strain in mice, hamsters, and rhesus macaques. We show that SC-Ad6-EBOV gp induces high serum antibodies after

single intranasal or intramuscular immunization and the production of binding and neutralizing antibodies in rhesus macaques. We show that SC-Ad6-EBOV gp mediates protection against pseudo-challenge. These data suggest that SC-Ad6-EBOV gp is a potent vaccine and may provide value during future EBOV outbreaks.

518. A Novel Gene Immunotherapy for Chronic HBV and HDV: Blocking Viral Entry by Neutralizing PreS1 Antibodies

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Background: In chronic hepatitis B and D virus (HBV/HDV) infection the virus evades host immune responses by inducing a severe T cell dysfunction/tolerance, and by secreting HBV surface antigen (HBsAg) to inhibit HBsAg-specific neutralizing antibodies. As the infectious HBV virions, in contrast to circulating HBsAg, predominantly display the PreS1/2 proteins, the presence of PreS1 antibodies can block viral entry and spread of infection. To circumvent, rather than to overcome or break, immunological tolerance in chronic HBV, we used the large HDV capsid antigen (HDAg) as a heterologous carrier of T cell help. Thus, genetic immunization with HDAg, linked to PreS1 sequences, will prime healthy T cells that support B cell activation and production of neutralizing PreS1 specific antibodies also in a host with chronic HBV. **Methods:** A total of 10 different HBV/HDV gene constructs containing various combinations of PreS1 sequences fused to HDAg genotype (gt) 1 and gt2 sequences were injected in C57BL/6 and BALB/c mice or rabbits by intramuscular immunization followed by *in vivo* electroporation. The DNA vaccine candidates were evaluated for the induction of both antibodies and specific T cells by ELISA and ELISpot. The elicited antibodies were further evaluated for their ability to neutralize HBV by an *in vitro* neutralization assay. **Results:** We found that the HDAg-induced T cells are genotype specific in both C57BL/6 and BALB/c mice, suggesting that the vaccine needs to contain both HDV gt1 and gt2 sequences. The various vaccine candidates differed in their ability to induce broadly cross-reactive PreS1 antibodies and to neutralize HBV *in vitro*. We have identified one vaccine candidate able to induce high levels of PreS1 specific antibodies which cross-reacted with the HBV genotypes A-F. Importantly, the vaccine-primed high levels of PreS1 antibodies in mice and rabbits that effectively neutralized HBV *in vitro*. Antibodies to multiple distinct individual PreS1 epitope specificities were induced. **Conclusion:** The current therapeutic vaccine candidate, using a strategy of circumventing immunological tolerance by inducing healthy heterologous T cells, induced functional and neutralizing HBV- and HDV- specific immune responses. This is a novel gene immunotherapy that has the potential to induce long-term off-therapy responses in chronic HBV, and possibly also in HBV/HDV co-infection.

519. Use of Pseudotyped, Replication-Deficient Influenza to Facilitate Testing of Gene Delivery Strategies for Treatment of Highly Infectious Diseases

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Infectious disease pandemics, causing thousands of deaths, are an important target for the development of new vaccines and treatments. One approach, administering broadly-neutralising antibodies (bnAbs) from vaccinated volunteers to provide passive immunity, suffers from costly antibody manufacturing, and the relatively short half-life of antibody in the circulation. This has resulted in the evaluation of strategies to deliver bnAb-encoding genes to provide passive immunity that is longer-lasting. In the case of highly infectious, lethal diseases, however, new treatment testing is hindered by restricted access to safe, bio-containment facilities. To address this issue, we have developed a strategy that facilitates testing in standard, readily available, Level 2 facilities. We generated novel, replication-deficient influenza viruses by replacing the haemagglutinin (HA) coding sequence with firefly luciferase transgene. Such viral configurations fail to propagate *in vitro*, but when HA sequences are provided *in trans*, efficient viral production occurs generating viral 'Fluciferase' particles capable of a single-round of infection. Upon administration to mice, the Fluciferase virus can be repeatedly visualised with non-invasive bioluminescence imaging in live animals, acting as a marker of infection. A prototype Fluciferase with the well-known A/PR/8/1934 influenza genetic background was generated. Pseudotyping this with the PR/8 H1 sequence generated H1-PR8 Fluciferase, which expresses luciferase in cell culture that can be blocked in a dose-dependent fashion by T1-3B neutralising antibody. Intranasal delivery of H1-PR8 Fluciferase to mice led to dose-dependent luciferase expression in the lung, which peaked at 24 hours post-delivery and lasted for at least 5 days. H1-PR8 Fluciferase infectivity in mice was also blocked by T1-3B antibody ($p<0.05$), at doses known to confer full protection (n=6/6 survival, $p<0.001$) against a lethal challenge of wild type H1N1 A/PR/8/1934 influenza. Pandemic influenza outbreaks of H5N1 and H7N9 bird flu strains, are predicted in the near future, but experiments involving these strains are complicated in terms of bio-safety. Using our approach, H5 and H7 Fluciferase strains were generated by supplying H5 and H7 *in trans*. Encouragingly, and similar to the prototype H1-PR8 Fluciferase, entry was also blocked by known neutralising antibodies in mice for both H5 Fluciferase (PBS-treated, 1.0E6±1.7E5 Relative Light Units (RLU), antibody-treated, 2.0E5±2.7E4 RLU; $p<0.05$) and H7 Fluciferase (PBS-treated, 2.9E6±4.4E5 RLU, antibody-treated, 4.7E4±1.3E4 RLU; $p<0.05$). Although the Fluciferase approach does not fully replace influenza challenge experiments due to its non-replicating feature, it can serve as a useful screening tool to bridge between cell culture and expensive wild-type challenge studies. This work demonstrates the utility of the Fluciferase model as a simple, cost-effective approach to evaluate new treatments for protection against highly infectious diseases, such

as Ebola virus, Coronavirus (MERS-CoV/ SARS-CoV), Marburg virus, Lassa virus and Nipah virus, etc., in standard, low containment facilities.

520. B-Cell Lineage Restricted eCD4-Ig Expression for HIV Prophylaxis via Lentiviral Vector Mediated CD34 Cell Transduction

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AAV-based gene delivery for the long-term expression of HIV broadly neutralizing antibodies (bNAbs) has shown potential for sterilizing protection from HIV-1 challenge in non-human primate (NHP) and humanized mouse models. Protection from bNAb resistant HIV strains with IC_{80} greater than 5μg/mL requires high levels of circulating bNAbs, yet non-immune expression predisposes to the development of anti-bNAb antibodies that may abrogate effectiveness. eCD4-Ig is a potent and broadly neutralizing HIV entry inhibitor composed of domains 1 & 2 of CD4 fused by an IgG Fc backbone to a CCR5 sulfo-mimetic peptide. AAV-delivered eCD4-Ig delivery to NHP myocytes resulted in stable serum levels from 17-77μg/mL for 40 weeks, with protection from 6 escalating SHIV-AD8 dose challenges. While less immunogenic than other studies with AAV-delivered anti-HIV bNAbs, eCD4-Ig did generate anti-inhibitor responses in all NHPs. AAV driven immunogenicity has been previously associated with ectopic over-expression. Efforts to mimic natural bNAb production via B cell specific expression may address this issue, while localizing eCD4-Ig production to the site of virus replication may lower required levels for protection. Here we report preliminary efforts establishing lentiviral vector systems to produce eCD4-Ig and induce tolerance through B-cell lineage restriction. eCD4-Ig producing LVs were generated with expression driven by either the constitutive elongation factor alpha promoter (EF1α) or the immunoglobulin β promoter combined with the immunoglobulin μ enhancer (EμB29). Transduction studies of pro-B (REH), pre-B (Nalm6), immature B (Ramos) and mature B (Raji) cell lines, along with T-cell lines (SupT1) and primary cells (CD34 HSPCs) demonstrated lineage specificity, with eCD4-Ig expression from EμB29 vectors increasing with B lineage development. Production levels *in vitro* ranged from 0.12-0.15 pg eCD4-Ig/cell/day, and was maintained for at least 70 days post-transduction. *In vitro* studies were conducted to examine the risk of possible heterodimerization with native IgG generated in B cell progeny from transduced CD34 cells. Co-transfection of the bNAb VRCO1 heavy & light chain with eCD4-Ig in HEK 293T cells demonstrated a significant proportion of generated eCD4-Ig/VRCO1 heterodimers, which may reduce eCD4-Ig effectiveness. Thus, novel disulfide or knob-in-hole (KiH) mutations were introduced into the CH3 domain of the Fc portion of eCD4-Ig to force homodimerization and disfavor heterodimerization. Co-transfection with VRCO1 showed both changes reduced heterodimerization, but only KiH mutations maintained eCD4-Ig native folding and anti-HIV activity. Finally, preliminary humanized mice experiments are underway using poly-cistronic eCD4-Ig/GFP expressing vectors to transduce umbilical cord blood derived CD34 HSPCs followed by engraftment in NSG mice. GFP expression in peripheral blood was observed primarily in CD33 myeloid cells with EF1α vectors, but primarily in CD19 B cells with EμB29 vectors. Serum eCD4-Ig was detectable in most mice (7/10) (~1ng/mL) 6 weeks post engraftment, with greatest levels observed 9 and 12 weeks post engraftment (1-5ng/mL) in EμB29 regulated eCD4-Ig mice. These preliminary *in vitro* and *in vivo* results support the potential for B cell lineage restricted expression of eCD4-Ig as a strategy for increasing anti-HIV potency and evaluating tolerance. Ongoing studies will investigate eCD4-Ig anti-HIV effect with cell-cell contact *in vitro*, HIV challenge in eCD4-Ig expressing NSG humanized mice, and tolerance studies in mice.

521. Incorporation of Immunostimulatory Property to Messenger RNA Molecule by Hybridizing RNA to Poly a Sequence for Effective Vaccination

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Antigen messenger (m)RNA-based vaccines have recently attracted much attention due to their safety, capacity to induce cellular immunity, and the flexibility of antigen design. On the other hand, immunostimulatory adjuvants for mRNA vaccines have yet to be vigorously studied. Herein, we attempted to incorporate the adjuvant functionality to mRNA molecules by introducing immunostimulatory double stranded (ds)RNA structure to mRNA through hybridization with complementary RNA. This strategy has several advantages, such as antigen protein expression and the immunostimulation being obtained simultaneously in the same antigen-presenting cell. Additionally, our system is comprised of only RNA and thus has few safety concerns. Finally, the double stranded mRNA can be encapsulated in various carriers, allowing delivery without mRNA degradation. In this strategy, mRNA translational activity should be maintained after RNA hybridization. Thus, we prepared mRNA introduced with dsRNA structure only in its poly A region, by hybridizing poly U RNA (mRNA:pU), as well as mRNA hybridized with full length of antisense RNA (mRNA:f-asRNA). The sense strands with 5' cap and uncapped antisense strands were prepared by in vitro transcription and were hybridized to each other at an equimolar ratio. After addition to dendritic cell (DC)-derived DC2.4 cells, both of mRNA:pU and mRNA:f-asRNA induced strong immunostimulation in quantitative PCR of pro-inflammatory transcripts. In reporter assay using luciferase mRNA, translational activity of mRNA:pU remained comparable to that of unhybridized mRNA, whereas mRNA:f-asRNA showed drastic decrease in protein translation efficiency. Introduction of mRNA:pU led to enhanced cell surface expression of DC activation markers, CD86 and CD40, in mouse primary DCs and human derived DCs in vitro, whereas unhybridized mRNA failed to activate these DCs. Mechanistic analyses indicated the involvement of Toll-like receptor (TLR)3 and RIG-I, dsRNA receptors, in the immunostimulation by mRNA:pU. Especially, RIG-I-mediated recognition of triphosphate groups in the uncapped 5' end of antisense strands was suggested to play

a critical role. Finally, mouse vaccination experiment was performed using ovalbumin (OVA) as a model antigen. OVA mRNA was injected to inguinal lymph nodes without using carriers. mRNA:pU induced efficient proliferation of DCs in the lymph nodes in histological sections. This result revealed high immunostimulatory property of mRNA:pU also *in vivo*. 7 days after the vaccination, mRNA:pU injection increased the number of OVA-reactive splenocytes producing IFN-γ in ELISpot assay, and also increased serum concentration of anti-OVA IgG, whereas unhybridized mRNA failed to show such responses. These results indicated that enhanced immunostimulatory property of mRNA:pU led to the efficient induction of antigen-specific cellular and humoral immunity after vaccination. In conclusion, the mRNA:pU formulation is a safe and efficient platform to boost the effect of mRNA vaccine, which can be loaded into various mRNA carriers.

522. An Optimized DNA Encoded Monoclonal Antibody (DMAb) Prevents Zika Virus Infection and Severe Testicular Atrophy in Mice

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Over 2 billion people globally are at risk of Zika virus (ZIKV) infection. Monoclonal antibody (mAb) clones isolated from human ZIKV patients have demonstrated protection against ZIKV in mouse and non-human primate animal models. Usage of MAbs for the prevention of viral infections is fast and can be effective, however, manufacturing, delivery and storage and temperature stability, pose significant limitations and economic hurdles on their deployment. Accordingly, at-risk global populations in developed and developing countries can remain without access to these highly promising therapeutic options. Our team has developed a novel strategy for the facilitated delivery of synthetic DNA-encoded mAbs (DMAbs) directly *in vivo*. This strategy utilizes CELLECTRA-EP® technology to deliver transient DNA encoded immunoglobulin (Ig) transgene to skeletal muscle for *in vivo* production and secretion. Using this approach, we engineered DMAbs expressing mAb ZK190, a highly potent, neutralizing clone that binds uniquely to the ZIKV E protein. We evaluated both DMAb-ZK190 and variant DMAb-ZK190-LALA, designed to abrogate FcR binding. In vivo we achieve peak levels of expression over 27.0 μg/mL and 62.1 μg/mL, respectively with persistent IgG expression detected past 10 weeks. These levels ultimately lead to 100% protection in a stringent lethal murine challenge model. Additionally, in a low dose challenge, ZK190 and ZK190 LALA DMAb treated mice display normal testes histology with no viral load, while untreated mouse testes are damaged by the lingering effects of ZIKV. We now have biologically relevant expression levels of both DMAbs in NHP models that will allow further testing in Zika NHP challenge models. Furthermore, we are evaluating the N-glycan profile of these DMAbs *in vivo* for their potential influence on Ig effector function. DMAb technology for infectious disease offers an important and possibly dramatic new option for global MAb treatments.

523. Functional Characterization of In Vivo Expressed DNA-Based Monoclonal Antibodies (dMAbs) against Respiratory Syncytial Virus (RSV)

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Respiratory Syncytial virus (RSV) is a major threat to the health of young children and elderly adults, and complications involved with lower respiratory infections can lead to hospitalization, and some patients may succumb to disease. Almost the entire population is infected with the virus during the first two years of life, but immunity in many cases is not sustained nor completely protective against subsequent exposure. Even though a vaccine remains an unmet need, passive immunization with an immunoprophylactic anti-RSV-F antibody (Pavilizumab) has successfully reduced hospitalizations in vulnerable infants. However, the use of this monoclonal Antibody (mAb) is limited to high resource settings and unavailable to the majority of the global at risk populations. While mAbs have been shown to be effective in providing protection against many infectious diseases their widespread use is limited. The limited *in vivo* half- life means multiple doses are required to maintain immunity, and the high costs and complexities involved in development, manufacture and cold chain distribution also hinder their global use. In response, new strategies based on the *in vivo* delivery of antibody genes are being developed. One such platform is dMAb, a synthetic plasmid DNA-encoded mAb In proof-of principle studies dMAbs have provided protection against various infectious diseases, including influenza, *pseudomonas* and Ebola in pre-clinical animal models. Here we describe an engineered anti-RSV-F dMAb. *In vivo* delivery of this dMAb resulted in robust systemic levels of the antibody in the serum of mice. Equivalent levels have been associated with protection from lower respiratory disease after RSV infection. In cotton rats, which is the gold-standard to model human disease following RSV infection, we observed maintained serum-expression of the dMAb up to 60 days after delivery. The antibody was also detected in lung-lavage samples, demonstrating effective biodistribution. Furthermore, serum from animals harboring RSV-F dMAb was functionally active in terms of antigen binding and neutralizing live virus. Our findings support the significance of dMAb as a viable platform technology to permit global access to monoclonal antibody-based immunoprophylactics to tackle the burden of infectious disease.

524. Replicating Single-Cycle Adenovirus Vaccine against *Clostridium Difficile*

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The emergence of a highly virulent strain of *Clostridium difficile* (*C. difficile*) in the early 2000's led to a dramatic increase in the severity, morbirty, and mortality of *C. difficile* infection (CDI). In 2014, an estimated 606,058 (439,237 initial and 166,821 recurrent) episodes of CDI occurred in the United States making it the number one hospital acquired infection. Those infections were responsible for an estimated 44,500 deaths *C. difficile* is an anaerobic, gram-positive, spore-forming bacillus. CDI typically develops when antibiotic treatment disrupts host commensal microbiota. CDI is characterized by the colonization of the large intestine in susceptible individuals. Once colonization has occurred, symptoms arise from the release of two toxins, Toxin A (TcdA) and Toxin B (TcdB), that are able to cause colitis that can result in life-threating disease. Standard CDI therapies utilize antibiotics that are limited by their broad spectrum and cause further disruption of the intestinal microbiota, which results in a high rate of recurrence (approximately 25%). One new approach to CDI treatment that has shown some promise in clinical trials is fecal transplantation; however, most studies using fecal transplants administered them using invasive delivery methods such as gastroscopy, nasogastric tube, or colonoscopy. While new treatments are certainly needed and useful, vaccines have historically been the most effective means to fight infectious diseases and can be delivered using far less invasive methods. For CDI, a vaccine may offer the best opportunity for sustained, long-term protection. Towards that goal, we developed a novel single-cycle adenovirus (SC-Ad) -gene-based vaccine against *C. difficile*. Unlike common replication-defective Ad vaccines, SC-Ad vectors replicate antigen genes thousands of times to amplify immune responses. However, unlike fully replication-competent Ads, SC-Ad cannot cause dangerous adenovirus infections. Low seroprevelance SC-Ad6 was engineered to express fused and secreted receptor-binding domains from *C. difficile*'s two endotoxins TcdA and TcdB. Western blot analysis of transduced mammalian cells demonstrated that the vaccine produced and secreted both TcdA and TcdB as fused and cleaved proteins. A single intramuscular or intranasal immunization with this SC-Ad vaccine generated TcdA and B binding antibodies that climbed over one half of a year to reciprocal titers above 10^5 against both toxin A and B. Samples collected 6 months after single immunization had an average reciprocal TcdB neutralizing titer of 160. When mice were challenged with a lethal dose of TcdA ten after the single-immunization, all PBS and control SC-Ad vaccinated animals succumbed to the toxin within 48 hours. In contrast, all animals in the SC-Ad *C. difficile* vaccine group survived. These data suggest that this replicating single-cycle Ad vaccine may have utility as a single mucosal or systemic vaccine against *C. difficile* and CDI.

525. Adeno-Associated Viral Vectored Delivery of a Novel Monoclonal Antibody Gene against Blood-Stage *Plasmodium Falciparum* Malaria

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Plasmodium falciparum malaria remains one of the most devastating infectious diseases, resulting in the death of more than half a million individuals every year. Despite extensive efforts, the development of an efficacious *P. falciparum* vaccine has proved exceedingly difficult, and new and innovative intervention strategies are likely to be needed. Following parasite release from the liver, blood-stage vaccines aim to reduce mortality, clinical disease, and transmission, whilst potentially also allowing for natural boosting of vaccine-induced responses and the acquisition of natural immunity. However, a significant challenge in the development of a blood-stage malaria vaccine is the need to induce, and maintain, the very high levels of antibodies necessary to neutralise the parasite's rapid invasion of red blood cells. An alternative approach to obtain the required humoral immunity against blood-stage malaria is to use potent monoclonal antibodies (mAbs) as prophylactics, which would bypass the need for a vaccine to aid in current malaria elimination programmes. Vectored immuno-prophylaxis (VIP) uses viral vectors such as adeno-associated virus (AAV) to deliver mAb-expressing genes, which are expressed *in situ* in virally transduced cells following immunisation and released into the plasma. The interaction between *P. falciparum* Reticulocyte-Binding Protein Homologue 5 (PfRH5) and its host receptor basigin has been shown to be essential for erythrocyte invasion by *P. falciparum*, and antibodies against PfRH5 that block this interaction profoundly inhibit the growth of the parasite. Recently, we isolated a panel of human mAbs targeting PfRH5 from vaccinated volunteers. In this study, we deliver novel, fully human, potently neutralising anti-PfRH5 mAbs by VIP in mice, and we achieve durable and high-level serum mAb expression that is strongly inhibitory of *P. falciparum* growth. This approach, combined with anti-malarial drugs and other control interventions, could provide an effective strategy towards the ambitious objective of malaria eradication.

526. In Vivo Delivery of Epitopes-Encoding mRNA: Differential Role of Vehicles on the Quality and Location of T Cell Responses

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The efficacy of immunotherapy relies heavily on targeting as many disease-relevant T cells as possible whether the goal is to promote immunity or tolerance. Antigen dose, route and frequency of antigen administration, the type of antigen-presenting cells (APCs) implicated, and the use of adjuvants or immunomodulators all contribute to the quality of the T cell responses. In addition, appropriate delivery systems are needed to protect antigens or antigen-encoding vectors

from degradation and clearance and to achieve better targeting of lymphoid tissues for maximal encounter between presented antigens and T cells. An epitope-based approach makes it possible to incorporate major epitopes across multiple antigens along with unique neoepitopes. Multiple epitopes expressed from a single DNA or mRNA construct can be differentially targeted for efficient presentation to both CD4 and CD8 T cells (Dastagir et al., Mol. Ther. Methods Clin. Dev. 2016; 4:27-38) and co-expression of different epitopes enables linked cross-regulation between the specific T cells. As a vector, mRNA offers the most efficient platform to manipulate dendritic cells (DCs) ex vivo and to conveniently co-express antigens and immunomodulators to render the APCs more immunogenic or tolerogenic. However, the potential of mRNA to target endogenous APCs for the purpose of tolerance induction has not been explored. Here, we compared two approaches to deliver mRNA encoding epitopes from several beta-cell antigens recognized in Type 1 diabetes: 1) a nanoparticle-based approach to target endogenous APCs in vivo (NP-mRNA) using a cationic lipid-based formulation, and 2) a cell-based approach whereby DCs, as exogenous APCs, are modified ex vivo by electroporation (DC-mRNA). The NP-mRNA formulation protected and facilitated efficient entry of mRNA into various endogenous APCs (DCs and macrophages, as well as stromal cells) within a wider network of lymphoid tissues compared to DC-mRNA. Indeed, using several mRNA-encoded reporter genes, we found that intraperitoneally injected DC-mRNA accumulated primarily in the pancreatic lymph nodes (PLNs), while NP-mRNA exhibited a broader distribution centered on PLNs and spleen, and extending to more distal lymph nodes. This biodistribution pattern was confirmed by assessing both CD4+ and CD8+ T cell responses in these tissues, using T cell adoptive transfers or MHC tetramer analysis. Antigen-specific T cells up-regulated CD25, Lag-3 and PD-1, and co-expressed IL-10 and IFN-γ to a different extent when stimulated by these two strategies. Moreover, co-delivery of immunoregulatory products also from mRNA was able to modulate the antigen-specific T cell responses. The successful targeting of lymph node stromal cells with our NP-mRNA was found to be of significant interest because of the tolerogenic potential of these non-professional APCs. In summary, we established efficient and versatile in vivo mRNA-delivery platforms for antigen-specific immunotherapy of immune-mediated diseases.

527. AAV-Mediated Expression of Monoclonal Antibodies Offers an Alternative Vaccination Strategy against Filoviruses

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AAV-mediated monoclonal antibody (mAb) expression is able to provide protective concentrations of mAbs in animal models for various infectious diseases, including Ebola virus. We have developed a novel AAV6 triple mutant capsid, AAV6.2FF, which facilitates rapid and robust mAb expression following intramuscular (IM) administration. 2G4, 5D2 and 7C9 are ZMapp generation mAbs and were the initial targets for our AAV6.2FF-mAb expression platform. AAV6.2FF-mAb expression following IM injection has been sustained for over six months. AAV6.2FF-2G4, AAV6.2FF-5D2 and AAV6.2FF-7C9 were administered to C57BL/6 mice as monotherapies at a dose of $1x10^{11}$vg per animal. Two weeks later, these animals were challenged with a lethal dose of mouse adapted Ebola virus (MA-EBOV) and the survival rates were 83%, 100% and 100% for AAV6.2FF-2G4, AAV6.2FF-5D2 and AAV6.2FF-7C9 respectively. A two-component cocktail of AAV6.2FF-2G4/AAV6.2FF-5D2 administered IM at $2x10^{11}$vg was 100% protective in mice when given 14 or 7 days prior to challenge. However, a three day lag period prior to challenge resulted in only 1/6 survival. Furthermore, AAV6.2FF-2G4/AAV6.2FF-5D2 was 100% protective when administered five months prior to challenge. Hence AAV6.2FF-mAb expression represents a viable alternative Ebola virus vaccination strategy. A comparison of AAV6.2FF-mAb expression between C57BL/6 and BALB/c mouse strains revealed a 40-50% greater mAb output from BALB/c mice than C57BL/6. AAV-mAb cocktail delivery poses the potential complication of co-transduced cells producing improperly paired heavy and light chains resulting in non-functional mAbs. Combined vectors administered as one IM injection was compared to separate administration of vectors in different flanks. Although not statistically significant, a decrease in mAb expression was observed in the combined vector administration group. In the mAb development field, importance is placed on the neutralization potency as a proxy for the overall effectiveness of the mAb. Despite this, using two different non-neutralizing mAbs (5D2 and 7C9) we were able to demonstrate 100% protection from MA-EBOV challenge. However, these vectors were engineering using murine IgG2a, which likely impacted their ability to functional effectively in the mouse model. The use of human IgG1 mAbs would allow for a smoother transition between preclinical models moving towards the clinic. The expression kinetics of AAV6.2FF-human IgG1 mAbs is currently being evaluated in BALB/c mice. Since the development of ZMapp, a second generation of potent mAbs that bind more critical epitopes of the Ebola virus glycoprotein have been characterized, which will further advance this vaccine platform. Additionally, the recent development of mAbs that target the Marburg virus glycoprotein allow the expansion of this vaccine platform to protect against Ebola virus and Marburg virus.

528. Therapeutic Macrophages Protect against Methicillin-Resistant Staphylococcus Aureus

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Bacterial infections continue to exert a tremendous burden on the public health system throughout the developing and developed world. Over-prescription of antibiotics, extensive antibiotic use in agricultural settings, and increasingly complex hospitalized patient populations undergoing treatment have fueled the rise of several highly antibiotic-resistant "superbugs", exemplified by methicillin-resistant *Staphylococcus aureus* (MRSA). Hematopoietic cells are endowed with a variety of mechanisms to control microbial pathogens. Macrophages in particular have long been appreciated as potent anti-microbial immune cells equipped with several receptors that allow for rapid recognition, phagocytosis, and killing of pathogenic microbes. Following recognition, macrophages also secrete inflammatory cytokines to orchestrate a robust multifaceted anti-bacterial immune response. However, macrophages function in a wide variety of other cellular processes as well including tissue homeostasis, wound repair, and in some cases immune suppression. Environmental cues from

neighboring cells via cell surface receptors as well as production of chemokines, and cytokines program macrophages to efficiently cater to the needs of the local environment. We, and others, find that human macrophages primed with pro-inflammatory cytokine interferon gamma (IFNγ) display enhanced killing of MRSA relative to unprimed cells. To investigate the utility of macrophages as a cell therapy for peritoneal MRSA infection, we injected IFNγ-primed murine bone marrow derived macrophages into infected mice and found a significant enhancement of survival in treated mice. Importantly, we also found that mice treated with human IFNγ-primed macrophages displayed significant improvements in survival relative to untreated controls. Our discoveries provide proof-of-concept for a novel macrophage cell therapy approach against methicillin-resistant *Staphylococcus aureus* and may offer a needed addition to our arsenal against infectious pathogens.

529. Evaluation of a Multivalent scFv-Fc DNA-Encoded Monoclonal Antibodies (dMAb™) Platform against Zika Virus (ZIKV) and Dengue Virus (DENV) Infections

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Zika (ZIKV) and Dengue (DENV) viruses are mosquito-borne flavivirus that cause from mild to severe pathologies varying from minor rashes to severe organ failure that could lead to death furthermore, infection by ZIKV specifically during pregnancy is associated with spontaneous abortion or severe developmental defects in newborns, including microcephaly and cognitive impairment that can be individually and societally burdensome. Previously published pre-clinical models have laid the rationale for using neutralizing monoclonal antibodies (mAbs) as basis for therapeutic intervention against ZIKV and DENV infections. While mAbs administration holds great promises as both prophylactic and curative approaches for infectious diseases there are conceptual and methodological impediments associated with the large scale administration of protein mAbs specifically for several millions people potentially at risk of contracting ZIKV or/and DENV infections. Over the past several years we have been developing an alternative approach based on the electroporation (EP) mediated of plasmid DNA-encoding monoclonal antibody (dMAbs) in skeletal muscles that leads to in vivo production and secretion of mAbs in the serum. In an attempt to design a more versatile dMAb platform, with increased pathogenic coverage, we have engineered two single-chain fragment variable-Fc (scFv-Fcs) dMAbs, Z-dMAb1-sc and D-dMAb1-sc that target ZIKV and DENV, respectively. We have also engineered an additional dMAb that encodes both Z-dMAb1-sc and D-dMAb1-sc in a multivalent bi-directional promoter format (Z/D-dMAb1-sc). We have used the CELLECTRA®-EP technology to deliver intramuscularly in a murine model various cocktail combinations of plasmid DNA encoding Z-dMAb1-sc and D-dMAb1-sc as well as individually formulated multivalent plasmid DNA encoding Z/D-dMAb1-sc dMAb. We observe that EP-mediated gene transfer of each of these scFv-Fc dMAbs leads to the secretion of functional scFv-Fcs in mice serum as assessed by ELISA and viral antigen binding assays. From this observation, we noted disparate scFv-Fc expression for Z-dMAb1-sc and D-dMAb1-sc when expressed in the single multivalent bi-directional promoter construct (Z/D-dMAb1-sc) compare to when the two DNA plasmid constructs were co-formulated in a single preparation or separately delivered at two-individual muscle sites. Furthermore, we also analyze how these various co-formulations and multivalent combinations affect the neutralization phenotype. Taken all together these data provide proof of concept for adopting a multivalent scFv-Fc dMAb platform that may prove more adaptable to combat infections by multiple pathogens such as ZIKV and DENV that are prevalent in overlapping endemic zones.

Metabolic, Storage, Endocrine, Liver and Gastrointestinal Diseases II

530. *Ex Vivo* Hepatocyte Reprograming Enables DNA Repair by Homologous Recombination to Correct Metabolic Disease after Cell Transplantation

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Ex vivo CRISPR-Cas9-mediated gene editing in hepatocytes using homology-dependent repair (HDR) is a potential alternative curative therapy to organ transplantation for metabolic liver disease. However, a major limitation of this approach in adult primary hepatocytes is that non-homologous end joining (NHEJ) is the predominant DNA repair pathway for double strand breaks. In this study, we hypothesized that *ex vivo* hepatocyte culture could reprogram hepatocytes to more strongly favor HDR after CRISPR-Cas9-mediated DNA double strand breaks. We determined optimal culture conditions for upregulation of genes involved in homologous recombination. RNA-Seq analysis demonstrated that within 24h of *ex vivo* culture, primary mouse hepatocytes begin to decrease hepatocyte metabolic functions and increase expression of genes related to mitosis progression and HDR. These differences became even more pronounced at 48h. Despite the downregulation of hepatocyte function genes, we found that hepatocytes cultured for 48h robustly engraft *in vivo*. In order to assess the functionality of CRISPR-Cas9-edited cultured hepatocytes long-term, primary hepatocytes from a mouse model of hereditary tyrosinemia type 1 (HT1) bearing a single point mutation were used. HT1 is an inherited liver metabolic disorder caused by a deficiency in the enzyme fumarylacetoacetate hydrolase (FAH). Hepatocytes from *Fah*$^{-/-}$ mice were isolated and transduced *ex vivo* with one AAV vector delivering both the Cas9 nuclease and a target guide RNA near

the SNP in the *Fah* gene (AAV-Cas9), as well as a second AAV vector delivering both a second guide RNA and a 1.2kb homology template (AAV-HT). *In vitro* analysis of the vectors in the isolated hepatocytes indicated that AAV-Cas9 induced robust cutting at the target locus. Additionally, upon delivery of AAV-HT, precise correction of the point mutation occurred by HDR after 48h. Corrected hepatocytes were then transplanted into recipient *Fah*$^{-/-}$ mice via splenic injection. The recipient mice were monitored and cycled on and off the protective medication, NTBC, until independent stable weight gain was achieved. NTBC-independence indicated the engraftment and robust proliferation of transplanted cells, which rescued the mice from liver failure. In conclusion, the data presented here highlight the potential of *ex vivo* hepatocyte gene editing after reprogramming to cure metabolic disease.

531. Curing Disease before Birth: *In Utero* Gene Therapy for the Treatment of Hereditary Tyrosinemia Type 1

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Introduction: We aimed to cure a murine model of human HT1 through *in utero* gene therapy and describe the biodistribution and genotoxicity of *in utero* lentiviral gene therapy in both mice and pigs. **Methods:** We performed direct fetal intrahepatic injections of a lentiviral (LV) vector carrying the human *FAH* gene under control of the liver-specific alpha1-antitrypsin promoter in *Fah*$^{-/-}$ mice. Injections were performed at day 15 of gestation with 10^7-10^8 TU/fetus. Correction of the diseased phenotype was confirmed biochemically, histologically and through the animals' ability to thrive off NTBC. Injections were repeated in wild-type mice using a LV vector carrying either the green fluorescent protein (GFP) or the luciferase gene under control of ubiquitous SFFV or CMV promoters. These mice were evaluated through immunohistochemistry (IHC) or bioluminescence imaging. Direct fetal intrahepatic injections of LV-GFP were then performed in a sow at day 65 of gestation using 10^8-10^9 TU/fetus. Here, biodistribution data was evaluated through PCR analysis. Injections were repeated in two *FAH*$^{-/-}$ gilts using LV-FAH. **Results:** Fourteen treated *Fah*$^{-/-}$ pups have demonstrated maintenance of healthy NTBC-independent growth curves after weaning, with 10 (71%) not requiring any NTBC cycling. These data correlate with liver repopulation by FAH-positive hepatocytes seen on IHC at one month post-weaning, as well normalization of tyrosine levels in these animals. No evidence of GFP expression was found through IHC analysis of maternal tissues in mice. Luciferase expression in mothers was limited to the uterus, and in pups luciferase was preferentially expressed in the liver. Preliminary real-time PCR analysis performed on fetal and maternal pig tissues after LV-GFP delivery demonstrated no evidence of lentiviral vector integration in maternal tissues, and vector presence only in liver and kidney in piglets. Two *Fah*$^{-/-}$ pig gestations treated with *in utero* LV-FAH gene transfers are proceeding with no complications and are due to farrow in one month. **Conclusion:** In a murine model of human HT1, *in utero* gene therapy using a lentiviral vector carrying the *FAH* transgene is curative, with complete repopulation of the liver with FAH-positive hepatocytes one month after weaning. *In utero* gene transfer with this method does not expose the mothers to any significant amount of the lentiviral vector in a large animal model.

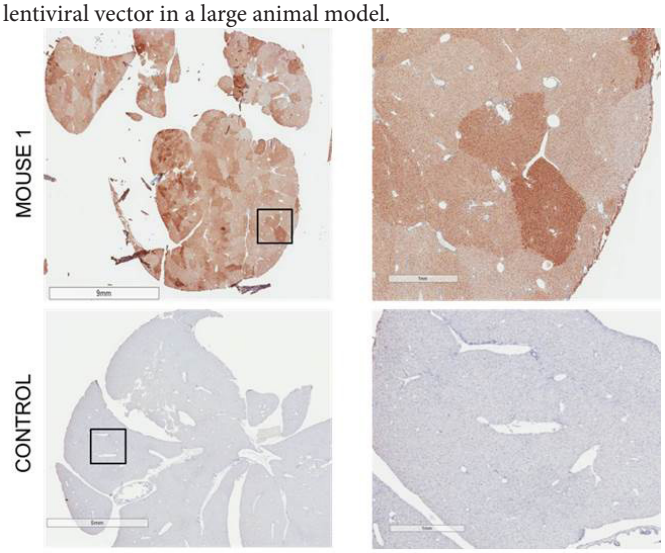

Figure 1. Complete liver repopulation with FAH-positive hepatocytes after *in utero* lentiviral gene transfers in mice.

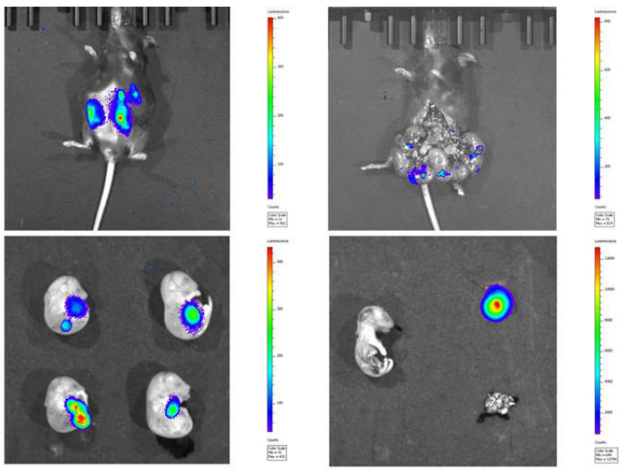

Figure 2. Bioluminescence imaging after *in utero* delivery of LV-Luciferase shows expression limited to the fetal liver.

532. The Efficacy and Safety of scAAV9/AGA Gene Therapy in Aspartylglucosaminuria Mice

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Aspartylglucosaminuria (AGU) is an autosomal recessive lysosomal storage disease caused by dysfunctional lysosomal enzyme aspartylglucosaminidase (AGA), resulting in the accumulation of the AGA substrate (aspartylglucosamine, GlcNAc-Asn) in a variety of tissues and body fluids. AGU patients show a slow but progressive phenotype characterized by intellectual disability, skeletal

abnormalities, and early mortality. Currently, there is no approved treatment for AGU. Due to the failure of bone marrow transplantation and the lack of feasibility of enzyme replacement therapy in this disease, gene therapy has become a reasonable and meaningful approach which might provide a long term therapeutic benefit to AGU patients. To test our hypothesis that scAAV9/AGA gene therapy may impart a therapeutic benefit to AGU mice, a comprehensive efficacy experiment was carried out. In this experiment, both male and female AGU mice were injected intravenously (IV) or intrathecally (IT) with either high ($1x10^{12}$ or $1x10^{11}$ vg/mouse for IV or IT, respectively) or low ($2x10^{11}$ or $2x10^{10}$ vg/mouse for IV or IT, respectively) doses of scAAV9/AGA vectors at 6 months old (early-symptomatic cohorts) or 2 months old (pre-symptomatic cohorts). The readouts of this experiment included AGA activity and AGA substrate levels in tissues and body fluids, behavioral tests, small animal imaging, and histopathology. Our 6 months old early-symptomatic cohorts demonstrated a clear therapeutic benefit of scAAV9/AGA treatment to AGU mice: 1) dose-dependently increased and sustained AGA activity in serum and peripheral tissues (heart and liver) to a supra physiological level; 2) dose-dependent reduction of GlcNAc-Asn accumulation in both central and peripheral tissues (brain and liver) and body fluids (CSF, serum, and urine); 3) significant reduction of the substrate accumulation peak detected at 5.1 ppm from *in vivo* magnetic resonance spectroscopy of the brain; 4) significant restoration of function from open field tests when high doses of AAV9/AGA vectors were injected either IV or IT; and 5) dose-dependent prevention of Purkinje cell loss in the cerebellum of treated AGU mice. Our 2 month old pre-symptomatic cohorts are still ongoing and collected data have shown similar, if not better, therapeutic benefit. In parallel, a pilot safety experiment was started more than 6 months ago, in which a very high dose (i.e. $1x10^{12}$ vg/mouse for IT, 10x higher than our efficacious dose) of scAAV9/AGA vectors was employed. Neither abnormal neurological symptoms nor body weight loss have been noticed so far in our treated AGU mice. Taken together, all these results demonstrate that treatment of AGU mice with AAV9/AGA vectors is effective and safe, providing strong proof-of-concept evidence that scAAV9/AGA gene therapy should be considered for human translation.

533. Lipid Nanoparticle Pre-Treatment Improves rAAV Diffusion in the Primate Liver and Enables an Increase of Therapeutic Transgene Expression

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Recombinant adeno-associated virus (rAAV)-vectors have been successfully used in preclinical studies and in clinical trials to express therapeutic proteins in the liver. Transduction of a limited percentage of hepatocytes can be sufficient for expression of secreted proteins. However, most hepatic metabolic monogenic disorders caused by the deficiency of an intracellular enzyme or membrane transporter would require a high percentage of hepatocytes to be transduced to achieve a therapeutic effect. This can be routinely achieved in rodents, but has proven to be a significant hurdle in large animals hampering the feasibility of such liver directed AAV based gene therapies. The liver has a very high capacity to remove particles from the circulation. The cells from the reticuloendothelial system (RES) play a central role in this clearance process. Those cells can ingest and destroy foreign material and therefore constitute the first cellular barrier between the blood flow and the liver tissue. The saturation of the hepatic reticuloendothelial cells by nanoparticles or lipids has been shown to block uptake of particles from the circulation. We hypothesized that saturation of the RES could increase AAV-vector transduction and distribution in the liver, subsequently improving transgene expression, in large animals. Therefore, we explored the potential of pre-treatment with Intralipid, an FDA approved emulsion of soy bean oil, egg phospholipids and glycerin in non-human primates. **Methods:** Non-human primates (NHPs, n=2) tested negative for the presence of anti-AAV5 neutralizing antibodies were injected intravenously with Intralipid (2g/kg) one hour before intravenous administration of AAV5 -hFIX at a dose of $9.7x10^{12}$ gc/kg. A control group (n=2) was injected with AAV5 -hFIX at the same dose after prior treatment with PBS. The animals were followed for 8 weeks before sacrifice. The levels of hFIX transgene in the plasma were analyzed by ELISA and the AAV vector DNA and transgene RNA copies numbers in liver tissue were determined by QPCR. The presence of AAV vector DNA and transgene RNA was detected in liver tissue samples by fluorescent in situ hybridization (FISH).**Results:** After Intralipid pre-treatment, an increase in the levels of hFIX transgene expression was observed in the animals injected with AAV5-hFIX when compare to the control group (average of 3.5-fold). A similar increase was observed at the hFIX mRNA levels. Accordingly, the vector DNA copies numbers were higher in the liver tissues of the animals treated with Intralipid than in the control group (average of 2.6-fold). Remarkably, Intralipid pre-treatment resulted in an enhanced diffusion of AAV5 vector through the liver tissue. The percentage of transduced hepatocytes was up to 4-fold higher when Intralipid was used. No adverse effect of Intralipid on the immunity against AAV5 capsid proteins or the transgene was observed. In summary, our data demonstrate that a pre-treatment with a lipid emulsion prior to AAV5 administration improves significantly the efficacy of AAV vector delivery to the liver and enables a broader hepatic cell targeting throughout the tissue. This approach represents a valuable tool for liver-targeted gene therapies application.

534. Therapeutic Efficacy of VTX-801, an Optimized AAV Vector for the Treatment of Wilson's Disease

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Wilson's disease (WD) is a disorder of copper metabolism that can present with hepatic, neurologic, or psychiatric disturbances, or a combination of these. If untreated WD is a life-threatening condition. Recently, we have demonstrated that an adeno-associated vector (AAV) serotype 8 carrying the human ATP7B cDNA provides long-term

correction of copper metabolism in WD young male mice. However, the size of this vector genome (5.1 Kb) surpasses the optimal size of a packaged AAV genome, which represents a major drawback for its clinical application. Furthermore, the efficacy of this vector in female mice and in animals with advanced disease (12 weeks) is notably lower than in young male mice. In this work, our main objective was to develop an optimized version of the gene therapy vector for clinical use. To generate a smaller version of ATP7B, the first four metal binding sites (MBS) from the amino terminal region of the protein were deleted. An AAV vector expressing a mini ATP7B transporter (Δ57-486-ATP7B) gene under the control of a liver-specific promoter was produced. Additionally, a recently developed synthetic AAV vector, AAV-Anc80, was used to limit the potential impact of pre-existing immunity and to improve liver transduction. WD mice were treated at different stages of the disease, and 1 year follow up evaluated for up was carried out. The parameters evaluated included copper in urine,hematology andclinical chemistry (with specific focus on liver function). The AAV vector expressing the mini-ATP7B transporter gene showed superior therapeutic efficacy in animals of both genders and in animals with advanced disease than the vector expressing the full-length protein. Moreover, we found that WD gene therapy, in addition to restoring copper homeostasis and preventing liver damage, significantly improved a number of additional parameters including cholestasis and hematological alterations for more than a year. Our data demonstrates that gene therapy using a synthetic gene therapy vector provides long-term correction of copper metabolism and additional pathological aspects associated with copper accumulation in WD animals independently of gender and disease stage, making such approach a clinical candidate for further evaluation.

535. Improved Propionyl-CoA Carboxylase Alpha (*PCCA*) Alleles for the Study of Propionic Acidemia (PA)

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Propionic acidemia (PA) is an autosomal recessive metabolic disorder caused by mutations in either *PCCA* or *PCCB*. The products of these genes form the alpha and beta subunits of the enzyme propionyl-Co A carboxylase (PCC), a critically important mitochondrial enzyme involved in the catabolism of branched chain amino acids. Many PA patients present within the first few days to weeks of life with symptoms, and lethality can ensue if clinical recognition and treatment is delayed. Laboratory investigations show characteristic elevations of propionylcarnitine, 3-hydroxypropionate, and 2-methylcitrate (2MC). Milder patients can escape from early presentations, but remain at risk for metabolic decompensation and late complications, especially cardiomyopathy. All individuals with PA can experience high mortality and disease related morbidity despite nutritional therapy. The failure of conventional medical and dietary management to treat PA has led to the use of elective liver transplantation as an alternative approach to stabilize metabolism, and mitigate the risk of lethal metabolic decompensations. The beneficial phenotypic effects noted after successful liver transplantation have enabled gene therapy to be entertained as a promising potential treatment for patients with PA. We have previously reported the first successful systemic AAV gene therapy using *Pcca*$^{-/-}$ mice, which display complete neonatal lethality by 24-36 hours after birth, and have now focused on the generation of new models that might recapitulate the resultant multisystemic manifestations seen in many PA patients. We sought to improve upon a previously described hypomorphic *Pcca* mouse model that relies upon the transgenic overexpression of the human mutation, A138T, from the chicken beta-actin (CAG) promoter on the *Pcca*$^{-/-}$ background. The *Pcca*$^{-/-}$; TG $^{\text{CAG PCCAA138T}}$ mice, despite having low residual hepatic PCC activity, manifest normal growth and relatively preserved survival on a regular chow diet, but do demonstrate a biochemical response to adenoviral and AAV vectors. We wondered whether the overexpression of PCCA A138T subunit might be responsible for the milder phenotype displayed by these mice, and therefore used genome editing to make the orthologous *Pcca*^{A134T} allele, speculating that endogenous, and lower level, expression from the *Pcca* locus may accentuate disease severity. Like the *Pcca*$^{-/-}$; TG $^{\text{CAG PCCAA138T}}$ mice, *Pcca*$^{A134T/A134T}$ mice are viable, without obvious gross pathology, and are fully fertile (both sexes), but do exhibit increased plasma 2MC, a critical biomarker of PA. *Pcca*$^{A134T/A134T}$ mice were challenged with a high protein diet which resulted in a ~10x increase in the plasma 2MC compared to wildtype littermates (5 micromolar n=4 vs. controls 0.40 micromolar n=4, p=0.0005). We next treated adult *Pcca*$^{A134T/A134T}$ mice with ~ $4x10^{12}$ gc/kg of AAV2/8 CAG PCCA, delivered by the retro-orbital route, to determine if AAV gene therapy could induce a change in metabolites. One week after treatment, 2MC trended lower and is under assessment for further longitudinal correction. In addition to the knock-in allele, three additional mutations in exon 5 were recovered: c.402dupT p.Val135Cysfs*29, c.398_401del p.Gln133Leufs*41 and c.402_402+12del. Like the previously characterized *Pcca* deletion allele, the first two mutations appear to be neonatal lethal in the homozygote state while the c.402_402+12del has no obvious phenotype. The new Pcca mutations we have generated should be useful to model the spectrum of clinically important postnatal features of PA, such as dietary sensitivity to precursors, growth failure, metabolic instability, cardiomyopathy, and afford facile testing of new treatments, such as AAV gene therapy.

536. Safe-Harbor Targeting Genome Editing by CRISPR to Treat MPS I Mice with AAV Vectors

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Gene therapy is promising for treating lysosomal diseases due to its potential for a permanent, single-dose treatment. Currently, there lacks treatment protocols providing sustained therapeutic benefits with minimized risks for patients with lysosomal diseases. To this end, we designed two constructs: one encoding Cas9 targeting intron 1 of albumin locus, and the other encoding promoterless IDUA cDNA sequence. Two days after hydrodynamic injection of these two plasmids into mucopolysaccharidosis type I (MPS I) mice, only the mice receiving both plasmids (n=3) had significant higher IDUA enzyme activities in the liver (6.6 fold of wildtype levels). Deep sequencing showed that the %indels at the target locus was only 0.2%, which yielded substantial enzyme expression in 2 days. To further evaluate this strategy, the two constructs were packaged into AAV2/8 vectors,

and injected into neonatal MPS I mice. One month post-dosing, plasma IDUA activities reached 1,953 fold of wildtype levels in treated MPS I mice (n=11). More importantly, IDUA activities in the brain, heat, liver and spleen increased to 3, 100, 21 and 23 fold of wildtype levels. Further, glycosaminoglycan levels in these tissues including the brain reduced to normal levels. At 4 months post-dosing, we will assess neurocognitive behaviors, and perform histological analysis, and investigate on-target and off-target gene modification rates. Innovative outcome measurements including proteomics and metabolomics profiling will also be conducted. Results from this study will be directly applicable for developing a clinical protocol of CRISPR-mediated *in vivo* genome editing to treat MPS I patients. As a platform strategy, the proposed study is broadly significant in providing "proof of principle" evidence for treating other lysosomal diseases. (NIH grant U54NS065768 and P01HD032652)

538. Gene Therapy for Crigler-Najjar Syndrome with AT342, a Liver-Targeted AAV8-UGT1A1 Vector - Preliminary Safety and Efficacy Results from a Phase 1/2 Study (VALENS)

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Objectives: Crigler-Najjar (CN) syndrome is a monogenic defect of bilirubin conjugation due to the absence or reduced expression of bilirubin UDP-glucuronosyltransferase (UGT). Without appropriate therapy, children are at risk for irreversible bilirubin-induced brain injury (kernicterus). Although phototherapy (PT) and phenobarbital reduce bilirubin levels, most patients with uncontrolled hyperbilirubinemia eventually require liver transplant. Gene transfer with the UGT1A1 gene may offer an alternative treatment for CN patients. We report on the preliminary safety and efficacy results from the first-dose cohort of three CN patients in a Phase 1/2 interventional study (VALENS) with AT342, an investigational AAV8 gene therapy product expressing a *UGT1A1* cDNA under the control of a liver-specific promoter. **Methods:** VALENS is a randomized, open-label, ascending-dose, delayed-treatment control Phase 1/2 study to evaluate the safety and efficacy of AT342 in patients with CN. Up to three dose levels of AT342 will be evaluated with 3 treated subjects and one delayed-treatment control subject per dose cohort. Key patient entry criteria include age ≥1 years old, bi-allelic mutations of *UGT1A1,* and requirement for ≥6 hours PT daily. Patients who received or planned to receive partial or whole liver, or hepatocyte transplant are excluded. All patients receive prophylactic oral prednisolone for 16 weeks beginning one day prior to treatment followed by a tapering period at Weeks 9-16. The three subjects in the first-dose cohort receive $1.5x10^{12}$ vg/kg of AT342 via IV infusion. Daily illumination time (hours of phototherapy) is collected by subject diary reports after baseline irradiance measures and patient distance from their PT system was collected. Bilirubin levels are assessed by weekly sampling for 12 weeks followed by bi-monthly assessments for the duration of the study. Primary endpoints include safety assessments and change in bilirubin levels from baseline at Weeks 12 (on PT) and 18 (off PT). **Results:** Preliminary safety and efficacy results from the first-dose cohort of three CN subjects in VALENS treated with AT342 will be presented. **Conclusion:** Gene therapy with AT342, an investigational liver-targeted AAV8-UGT1A1 vector may be a potential treatment option for patients with CN aged ≥1 years old experiencing consistently high unconjugated serum bilirubin levels despite aggressive management with daily PT.

539. Gene Therapy for Argininosuccinic Aciduria

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Argininosuccinic aciduria (ASA) is the second most common genetic disorder that affects the urea acid cycle and is caused by deleterious mutations in the argininosuccinate lyase (ASL) gene. Total loss of ASL activity results in severe neonatal onset of the disease, characterized by hyperammonemia within a few days of birth, which can rapidly progress to coma and death. Current treatments for ASA are limited to dietary restriction, arginine supplementation, and nitrogen scavenging drugs, with severe onset of the disease currently being treated by orthotropic liver transplant. Adeno-associated viral (AAV) vector based gene therapy would provide a new treatment option, helping to alleviate the symptoms associated with urea acid cycle disruption by providing stable expression of the ASL protein in the liver. A murine hypomorphic model of ASA was used to determine the efficacy of AAV8 gene therapy. A codon-optimized version of the human *ASL* gene was packaged within the AAV8 capsid for targeted delivery to the liver of the ASA hypomorph mouse. Vector was administered intravenously into newborn mice within 24 hours of birth via the facial temporal vein at doses of 10^{10} and 10^{11} genome copies (GC)/mouse. Median survival increased from 22 days for untreated ASA hypomorph mice to 136 days and 165 days for the low and high vector doses, respectively. An increase in body weight was also observed in mice treated with the AAV8 vector compared to untreated mice. As untreated ASA hypomorph mice survived until weaning, we attempted an intravenous vector administration approach in adult mice. Adult ASA hypomorph mice average 9.5 g in body weight. AAV8 vector was administered intravenously by retro-orbital injection at doses of 10^{13} and 6×10^{13} GC/kg and mice were monitored for 3 months. Following vector administration, there was increased survival and body weight in all groups. Correction of both liver transaminase levels and the disease-associated metabolites, argininosuccinic acid and citrulline, were observed within the first month after vector administration. Our results indicate that gene therapy with an AAV8 vector is a viable approach for the treatment of ASA.

540. Sustained Correction of Phenylketonuria by a Single Dose of AAVHSC Packaging a Human Phenylalanine Hydroxylase Transgene

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A novel group of Clade F adeno-associated viruses have been isolated from normal human CD34+ hematopoietic stem cells (AAVHSCs) and have shown high-efficiency nuclease-free gene editing as well as gene transfer capabilities. In biodistribution studies in mice and non-human primates we have observed significant transduction of hepatocytes following intravenous delivery of AAVHSC demonstrating that AAVHSCs have tropism for mammalian liver. To evaluate whether a hepatotropic AAVHSC could be used to deliver a therapeutic gene and correct a disease-associated metabolic phenotype in the liver, AAVHSC15 was studied in PAH^{enu2} mice. These mice harbor a missense mutation (F263S) in the phenylalanine hydroxylase (*PAH*) gene resulting in less than one percent of wild-type levels of phenylalanine hydroxylase enzyme (PAH) activity, have 40- to 50-fold elevations in serum phenylalanine (Phe) on a normal chow diet, and are a model for the classic form of phenylketonuria (PKU) in humans. AAVHSC15 packaging a human *PAH* transgene driven by a ubiquitously expressing promoter (AAVHSC15-PAH) was prepared by triple transfection in HEK293 cells and purified through two rounds of CsCl density gradient ultracentrifugation. PAH^{enu2} mice were maintained on standard chow diet and had a constant serum level of Phe of ~2000 μM. Mice received a single intravenous (IV) injection of either vehicle alone or vehicle containing increasing amounts of AAVHSC15-PAH. Serum levels of Phe and of tyrosine (Tyr), the metabolic product of PAH activity, were analyzed weekly and tissues were harvested at various time-points for measurement of vector genomes and *PAH* mRNA by ddPCR, and PAH activity in liver. One week post-dose, the serum levels of Phe were normalized to less than 150 μM ($p<0.0001$) and the serum levels of Tyr were increased in animals treated with AAVHSC15-PAH ($p<0.0001$). These changes were associated with dose-dependent increases in human *PAH* vector genomes, human *PAH* mRNA, and PAH enzymatic activity in livers of treated animals. Durability of responses were dependent on dose of AAVHSC15-PAH, with sustained reductions in serum Phe out to >18 weeks post-dosing at the highest dose tested ($p<0.0001$). No changes in liver enzyme levels in sera were noted following treatment suggesting that AAVHSC15-PAH was well-tolerated at these doses. Neither changes in serum Phe nor hepatic PAH activity were observed in animals treated with vehicle alone. Vector sequences in AAVHSC15-PAH were optimized including the addition of a liver-specific promoter resulting in HMI-102. HMI-102 normalized serum Phe in PAH^{enu2} mice at ten-fold lower doses with durability in response seen out to >14 weeks in an ongoing study. Taken together, these data demonstrate that administration of AAVHSC-based gene transfer vectors packaging a human *PAH* transgene normalized serum Phe in PAH^{enu2} mice. The durable correction of serum Phe observed suggests that HMI-102 shows potential for further development as one-time, *PAH* gene replacement therapy for PKU in humans in the absence of dietary intervention.

541. Evaluation of an AAV8 Gene Therapy Approach in a Mouse Model of Maple Syrup Urine Disease

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Maple syrup urine disease (MSUD) is an autosomal recessive, metabolic disorder caused by a deficiency of the branched-chain alpha-keto acid dehydrogenase (BCKDH). This mitochondrial multi-enzyme complex is responsible for the oxidative decarboxylation of the branched-chain amino acids (BCAAs), leucine, isoleucine, and valine. Buildup of BCAAs in the body and their toxic by-products (ketoacids) in the blood and urine gives the disease its name due to the distinctive sweet odor of affected infants' urine (branched-chain ketoaciduria). The intermediate MSUD mouse model is a hypomorph, with the mouse version of the E2 subunit of BCKDH knocked out and a human version of E2 knocked in for low level expression. This mouse model displays elevated levels of the BCAAs, characteristic of MSUD, with increases in leucine, isoleucine, and valine of 10-, 9-, and 8-fold, respectively. Mice were administered intravenously with 10^{10}-10^{11} genome copies per mouse (GC/mouse) of an AAV8 vector expressing the human E2 subunit of BCKDH via the facial temporal vein within 24 hours of birth. A dose-dependent increase in survival and reduction in blood BCAAs was seen following vector administration. Dose-dependent expression of the E2 transgene was confirmed by immunohistological staining. Therefore, the continuous synthesis of the E2 subunit by the liver following systemic delivery of a gene therapy vector expressing E2 could be used to treat MSUD.

542. Whole-Body Therapy for Sandhoff Disease via Intravascular Gene Therapy

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Sandhoff disease (SD) is a lysosomal storage disorder that, due to a deficiency in the heterodimeric enzyme Hexosaminidsase (Hex), causes fatal neurologic disease in children. In mouse and cat models of SD, direct brain injection of adeno-associated viral (AAV) vectors expressing Hex has greatly ameliorated the central nervous system (CNS) disease component, leading to peripheral disease as the ultimate cause of death in many cases. In an effort to develop a less invasive therapeutic route that could more effectively treat the whole body, we utilized an intravascular (IV) delivery of a bicistronic AAV vector. As part of the evaluation of the efficiency of this therapy, ultrasound shear wave elastography (SWE), a novel imaging technique, was utilized. SWE uses propagation of shear waves to map tissue stiffness, yielding information about the presence or status of disease in an organ. Affected cats were treated at 1.5-2.2 months of age by cephalic vein injection of

an AAVPHP.B bicistronic vector expressing both Hex subunits at a dose of $1.5x10^{13}$ vg/kg body weight. Cats were followed with ultra-high-field MRI and MRS, serial physical and neurological examinations, routine blood work, and cerebrospinal fluid analysis. SWE was performed on each IV treated cat for comparison to normal and untreated controls. When treated cats reached humane endpoint, HexA specific activity was determined throughout the brain entirety and in 7 regions of the spinal cord using a fluorogenic substrate (MUGS). Brain HexA activity ranged from 0.4-10% of normal, which was not statistically different from untreated cats, though there was a trend towards significance in cerebellar regions with p-values <0.06. In the spinal cord, there was a significant difference ($p<0.05$) in HexA activity between untreated and IV treated SD cats in the cervical intumescence only. HexA activity was highest (~27% of normal activity) in the cervical and lumbar spinal cord regions. Increased survivability of IV treated (6.8±1.6 months) versus untreated SD cats (4.3±0.5 months) trended towards significance ($p<0.06$). Improved quality of life in IV treated cats was demonstrated by delayed onset of severe neurologic abnormalities, including ataxia, whole-body tremors, and inability to ambulate. SWE revealed a significant difference ($p<0.05$) in the elasticity of the liver between normal and untreated SD cats, without significant improvement in IV treated SD cats. This finding is supported by HexA activity in the liver, which was 15 +/- 10% of normal and not significantly different from untreated levels. Overall, these results demonstrate some restoration of Hex activity in the CNS and moderate amelioration of disease progression, which may be improved by using higher vector doses or alternative capsids. Recent results from related studies suggest that AAVPHP.B confers no benefit over conventional capsids in the cat CNS. Finally, SWE detected abnormal liver elasticity in SD cats and is a promising, noninvasive method to measure whole-body therapy for SD.

543. An *In Vivo* Enrichment Platform to Enhance Hematopoietic Cell-Directed Gene Therapy

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Hematopoietic stem cell (HSC) transplantation following viral modification of autologous product is being investigated as therapy for multiple inherited disorders. As one example, our FACTs team (FAbry disease Clinical research and Therapeutics) is investigating the safety of *ex vivo* lentiviral (LV) modification and infusion of patient HSCs to achieve long-term correction of Fabry disease (clinicaltrials.gov: NCT02800070). After engraftment, leukocytes differentiating from the genetically modified HSCs can secrete α-galactosidase A (α-gal A) and uncorrected bystander cells can take it up. This may lead to systemic enzymatic correction and substrate reduction in many tissues. Treatments such as these may benefit from a directed method to enrich for genetically modified cells after transplant. Such enrichment may lead to an increase in therapeutic cargo delivery. Enriching a mature compartment of modified hematopoietic cells rather than HSCs may be sufficient to elicit a therapeutic benefit in many settings. Targeting mature cells may also allow finer control and reversibility of enrichment with less potential side-effects. T cell and B cell proliferation and maturation are inhibited by mycophenolic acid (MPA). The pro-drug mycophenolate mofetil (MMF) is routinely used in the clinic as an orally administered immunosuppressant with few serious side-effects. The target of this drug, inosine-5'-monophosphate dehydrogenase 2 (IMPDH2), can be conferred with resistance by mutating two amino acids (T333I and S335Y; $IMPDH2^{IY}$). We have constructed dual-promoter LV vectors that engineer expression of $IMPDH2^{IY}$ and other transgenes of interest. We have been optimizing our enrichment platform using α-gal A as the model transgene. In an *in vitro* model, we have shown an 8-fold increase in vector copy number (VCN) after exposure of transduced cells to MPA. Enrichment also increased cellular and secreted α-gal A activity by 8-fold and 13-fold, respectively. We then engineered luciferase expression in our cell model (Luc+). We xenografted Luc+ cells in NOD/rag/gamma (NRG) mice to determined appropriate cell dosage and to track location and timing of engraftment. Next, we will xenograft cells expressing $IMPDH2^{IY}$ and administer MMF to optimize enrichment conditions. We have also initiated work with *ex vivo*-transduced syngeneic bone marrow transplants (BMT) in Fabry mice. Preliminary data from these studies suggest that the vector and this enrichment schema have no adverse effects on hematopoiesis or the health of mice. We hope to apply optimized MMF conditions from our xenograft model to our BMT model to examine any benefits of our enrichment platform. We anticipate that our strategy will have utility in many other inherited disorders treatable with HSC-directed gene therapy.

544. Post-Infusion Cell Enrichment: Gaucher Disease as a Model

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Hematopoietic stem cell (HSC) transplants are employed in many gene therapy clinical trials. Advantages of HSC transplants include their diverse differentiation capacity along with the use of autologous infusions to reduce the chance of rejection. Prior to infusion, HSCs can be modified *ex vivo* by lentiviral (LV) vector-mediated transduction resulting in therapeutic target integration into the patient's genome. Production difficulties may include: low HSC harvest from patients, inefficient transduction, inability to reach biologically-significant therapeutic levels of transgene expression, or poor engraftment. *In vivo,* transduced HSCs may become out-competed by non-transduced cell populations. Including an enrichment method for modified cells either *ex vivo* during production or *in vivo* post-infusion may be one

possible strategy to address these issues. Inosine-5'-monophosphate dehydrogenase 2 (IMPDH2) is required for B cell and T cell proliferation due to its role in *de novo* guanine nucleotide synthesis. Mycophenolic acid (MPA), orally administered as the prodrug mycophenolate mofetil (MMF), inhibits IMPDH2 activity. MMF is a commonly used immunosuppressant with minimal side effects when administered at relatively low doses. A mutant form of IMPDH2 containing two amino acid substitutions (IMPDH2^{IY}) confers resistance to MMF. B cells and T cells expressing IMPDH2^{IY} may have a selective growth advantage in the presence of MPA while other cell compartments should remain largely unaffected. We have previously used IMPDH2^{IY} enrichment *in vitro* in a Fabry disease model. To demonstrate the utility of the IMPDH2^{IY} enrichment 'platform' for multiple disorders, we will evaluate it in a Gaucher disease (GD) model. GD is a Lysosomal Storage Disorder caused by mutations in β-glucocerebrosidase (GBA). Functional GBA breaks down glucocerebroside into glucose and ceramide. Clinically, accumulation of glucocerebroside and its metabolite glucosylsphingosine can cause a spectrum of symptoms. While enzyme replacement and substrate reduction therapies exist for GD, the high cost, effect on quality of life, and side effects associated with life-long treatments are not ideal for patients. To evaluate the IMPDH2^{IY} platform for GD, we have constructed a dual-promoter LV that engineers expression of IMPDH2^{IY} and GBA. Preliminary experiments show over 10 times greater GBA activity in transduced cell populations. Enrichment of transduced cells will be monitored through cell viability counts, vector copy number determination, and GBA activity levels. The ability of this platform to enrich for transduced cells, regardless of therapeutic target, would show its versatility in gene therapy applications involving regulated expression of *ex vivo*-modified HSCs.

545. Liver-Based Expression of the Human Alpha-Galactosidase a Gene in a Murine Fabry Model Results in Continuous Therapeutic Levels of Enzyme Activity and Effective Substrate Reduction

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Fabry disease (FD), an X-linked lysosomal storage disease, is caused by mutations in the *GLA* gene encoding α-galactosidase A (α-GalA). FD is characterized by progressive systemic accumulation of the enzyme's substrates, globotriaosylceramide (Gb3) and lyso-Gb3, leading to renal, cardiac and/or cerebrovascular disease and culminating in premature patient demise. The disease is most commonly treated by enzyme replacement therapy (ERT). However, ERT requires a lifetime of biweekly infusions and may not clear all substrate from secondary organs. A more effective and long-lasting treatment would benefit FD patients. Thus, an AAV-mediated, liver-targeted gene therapy approach was evaluated in a knock-out mouse model for Fabry disease. An AAV-mediated GLA cDNA gene therapy was evaluated in a Fabry mouse model (GLAKO) that lacks α-GalA activity and accumulates high levels of Gb3/lyso-Gb3 in plasma and tissues. This strategy employs an episomal AAV vector encoding human *GLA* cDNA (h*GLA*) driven by a liver-specific promoter. Administration of six single doses of increasing amounts of AAV h*GLA* cDNA resulted in supraphysiological expression of plasma α-GalA (up to 50 fold of wild type) by day 14, was well tolerated, and was stable for 6 months post-injection. Dose-dependent increases in α-GalA activities were achieved in liver, heart, kidney and spleen with a corresponding reduction of Gb3/lyso-Gb3. An improved cDNA vector administered to GLAKO mice in a 2 month follow up study produced stable plasma α-GalA levels up to 200 fold of wild type. α-GalA activity in heart and kidney of mice treated with the improved cDNA averaged 20- and 3- fold over wild type levels, respectively, and Gb3/lyso-Gb3 in these tissues were near normal levels. Appropriate glycosylation of the α-GalA enzyme produced from liver cells was confirmed by *in vitro* experiments to ensure efficient mannose-6-phosphate mediated lysosomal uptake in target tissues. These studies provide "proof-of-concept" for AAV-mediated targeting of hepatocytes *in vivo* to express therapeutic levels of human α-GalA. The concomitant marked reduction in the accumulated Gb3/lyso-Gb3 in key tissues further support this liver-based AAV h*GLA* cDNA approach as a potential therapy for FD patients.

546. Non-Myeloablative Conditioning Regimen Using an Anti-Ckit Antibody for Hematopoietic Stemcell Targeted Gene Therapy for a Murine Model of Mucopolysaccharidosis Type II

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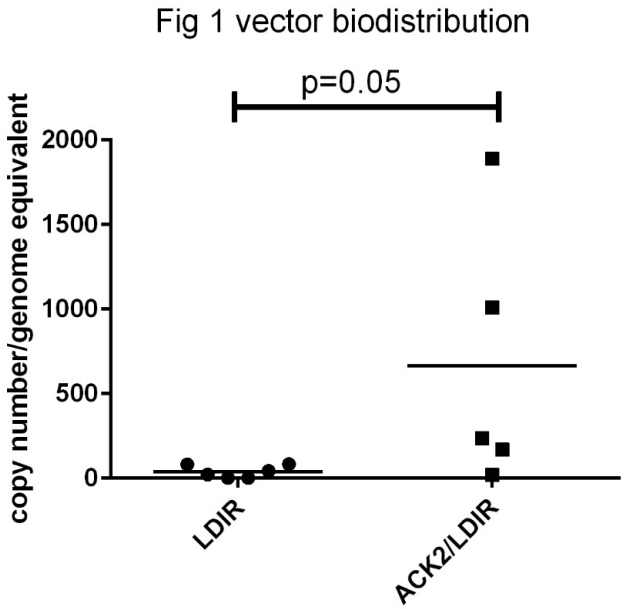
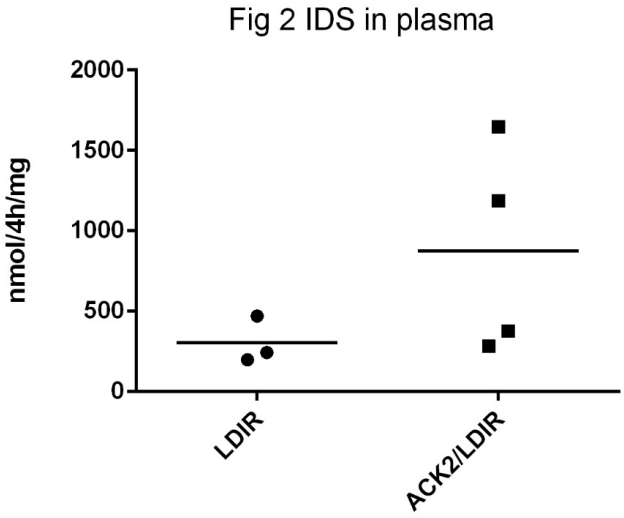
Introduction: Mucopolysaccharidosis type ll (MPS ll) is a lysosomal storage disease caused by a deficiency of iduronate-2-sulfatase (IDS) which leads accumulations of glycosaminoglycans (GAGs) in various tissues including brain. Althogh enzyme replacement therapy and hematopoietic stem cell transplantation (HSCT) are available, both therapies do not have any effect to brain disease of MPS II. Our previous study demonstrated that the hematopoietic stem cell targeted gene therapy had a certain effect of CNS disease of MPS II. However, it requires toxic preconditioning such as total body irradiations and strong chemotherapies. To avoid this limitation, we tested myeloablative conditioning preconditioning regimen for lentivirus mediated hematopoietic stem cell gene therapy for murine model of MPS II. We and others reported anti-ckit antibody (ACK2) combination with low dose irradiation conditioning resulted in very high donor chimerism in hematopoietic stem cell transplantation for other mouse models of lysosomal storage disease. Thus, we used this conditioning regimen for hematopoietic stem cell targeted gene therapy for MPS II. Methods: Whole bone marrow cells were harvested from humerus, femurs and tibias of donor MPSll mice(8-12 weeks old), and sorted into lineage-negative populations. Lineage-negative cells were transduced at a multiplicity of infection of 50 with lentiviral vector expressing IDS under control of MND promoter for 16-24h. Then, these transduced cells were infused intravenously to MPS ll mice in various preconditioning regimens including lethal irradiation, low dose irradiation (LDIR) and ACK2/LDIR combination. After gene therapy, we analyzed plasma IDS activity and copy number of vector in peripheral blood up to 16 weeks.

Results: After 16 weeks after gene therapy, vector copy numbers in ACK2/LDIR group were higher than those of LDIR group (Fig 1, p=0.05) .



In addition, IDS activities increased in the ACK2/LDIR group comparing to the LDIR group(Fig 2).



Conclusions:In this study, we demonstrated that the administration of ACK2 combination with LDIR might be useful as a preconditioning of HSC targeted gene therapy for MPS II.

547. Development of a Multi Domain Responder Index for Clinical Trials with Multi-Domain Diseases

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Objective: To develop and validate a Multi-Domain Responder Index (MDRI) for clinical trials with multi-domain diseases. Measuring clinical data from multi-domain disorders according to current standards is challenging due to the multi-faceted and heterogenous disease manifestations. To address these complexities, we devised a Multi-Domain Responder Index to accurately summarize the totality of data generated from a clinical trial. As a guide, we used a MPS IIIA clinical trial with bio potency, biophysical and neurocognitive evaluations. To assess the index in the subjects, we utilized a Natural History Study (NHS) as a control to validate the regressive slope as well as the standard error of the mean (SEM). Additionally, we created a nominal reaction score from the NHS in order to easily interpret the results. If the subject scored above the predicted NHS +/- SEM, they received a +1. If the subject scored within the NHS +/- SEM, they received a 0. If the subject scored below the NHS, they received a -1. These preliminary findings show the feasibility and applicability of our Multi-Domain Responder Index in supporting the totality of all of the Clinical Data generated from Clinical Trials. The MDRI demonstrates the comprehensive benefit of treatment compared to the natural history of the disease while simplifying multitudes of data variables into one coherent score.

548. Abstract Withdrawn

Neurologic Diseases (Including Ophthalmic and Auditory Diseases) II

549. Gene Therapy Restores Normal Weight in a Obese Bardet-Biedl Syndrome 1 (*BBS1*) Mouse Model

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The autosomal recessive Bardet-Biedl syndrome (BBS) is one of the best characterised ciliopathies and is associated with early onset

blindness, complex endocrine dysfunction, severe obesity, cognitive impairment and renal disease. Affected children will eventually go blind usually beginning in their first decade owing to progressive retinal degeneration. Within the first year of life they will gain an extraordinary amount of body weight which if unchecked will progress to life-threatening obesity, diabetes and high blood pressure. There is currently no cure for BBS, even when diagnosed early, symptom-based treatments will only manage unpreventable complications such as retinal degeneration and obesity refractory to dietary measures. Up to twenty genes has been found to be causative of BBS and the most common genes mutated in BBS patients is BBS1, accounting for 49% of patients. The most prevalent mutation in *BBS1* is the missence change M390R found in BBS patients worldwide. The human and mouse BBS1 amino acid sequences have a 93% of homology. The well known mouse model $Bbs1^{M390R/M390R}$ recapitulates the patients features including obesity. Both mutant males and females are 35% heavier than their wild-type littermates from early estages of development. It has been demonstrated that hyphothalamic neurons in $Bbs1^{M390R/M390R}$ animals are not able to translocate the leptin receptor (LepR) to the membrane and they have a reduction of the Pro-opiomelanocortin (Pomc) gene expression. Here we describe how the delivery of the Adeno-associated virus 2/8 (AAV2/8) expressing the human BBS1 cDNA, under the control of the constitutive promoter human elongation factor 1α-subunit (EFS) is able to target brain hypothalamic areas and restore BBS1 activity. Following a single injection of AAV2/8-EFS-hBBS1 in newborn pups (P0), both $Bbs1^{M390R/M390R}$ males and females show a reduction of weight gain from the beginning, with transgene expression lasting up to a year. Circulating leptin was assessed and the treated animals show a reduction in the levels of the leptin. However, unlike previous reports our data indicates that there are no differences in food intake between Wild-type, $Bbs1^{M390R/M390R}$ mutant and AAV2/8-EFS-hBBS1 $Bbs1^{M390R/M390R}$ treated mutant animals. We found specific expression of *hBBS1* in the retinae of $Bbs1^{M390R/M390R}$ animals treated with AAV2/8-EFS-hBBS1 a year after injection. We also injected AAV2/8-EFS-hBBS1 intravenously into $Bbs1^{M390R/M390R}$ animals at P0. Human BBS1 expression was detected in the retinae and brain for up to 12 months. Intravenously treated animals also showed a reduction of weight gain in both in males and females. In summary we found that gene therapy with AAV2/8-EFS-hBBS1 mediates early expression of the human BBS1 in the hypothalamus and retina and is able to regulate the weight gain in mutant $Bbs1^{M390R/M390R}$ animals.

550. Additive Effects of Intrathecal and Intravenous Combination Dosing of *CLN1* Gene Therapy in the Mouse Model of Infantile Neuronal Ceroid Lipofuscinosis

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Infantile neuronal ceroid lipofuscinosis (INCL), a lysosomal storage disorder caused by mutations in the *CLN1* gene, is a severe neurodegenerative disease affecting both the central nervous system, including the spinal cord, and peripheral organs, for which there is currently no cure or treatments that address the underlying cause. The onset of symptoms in patients with the classic presentation occurs between 6 and 24 months of age. Visual failure, cognitive and motor decline, and seizures progress rapidly until 3 to 5 years of age, when patients are disconnected from their environment and require intensive palliative care. Death usually occurs by 7 years of age. The INCL mouse model (*CLN1*-KO) recapitulates the major features of the disease, with neurological deficits appearing at 4.5 months and premature death occurring at 8 months of age. In the past we have presented our results with an intrathecal (IT) approach, delivering self-complementary AAV9 (scAAV9) carrying the *CLN1* gene directly into the cerebrospinal fluid (CSF) of INCL mice. With an initial low dose ($7x10^{10}$ vg), early treatment delayed the onset of symptoms and extended survival to double their expected lifespan, while treatment after symptom onset had no effect on survival. Using the same IT approach with a 10-fold higher dose ($7x10^{11}$ vg), early treatment conferred a normalized lifespan and significantly improved behavioral outcomes. Encouragingly, this higher dose also conferred some survival and behavioral benefits to early-symptomatic INCL mice, treated at 4.5 months. However, the maximum IT dose is constrained by the small volume that can be delivered into the CSF and limits on the extent to which AAV can be concentrated. In order to escalate the dose, the IT injection was combined with a systemic intravenous (IV) injection of the same vector, delivered sequentially. We hypothesized that combining IT with IV delivery, with a higher overall dose, would provide greater therapeutic efficacy, especially in older animals. Interestingly, we saw similar therapeutic effects when the same overall dose was administered, regardless of whether it was delivered IT or IV. When a maximal feasible IT dose was administered in conjunction with an injection of the same total vg IV dose ($7x10^{11}$ vg IT + $7x10^{11}$ vg IV), older symptomatic *CLN1* mice experienced improved survival and behavioral outcomes compared to IT or IV injection alone. The combination dosing has been well tolerated, with no adverse events observed in the treated animals. These studies are still ongoing, including testing the possible added benefits of treating younger pre-symptomatic mice. Ongoing clinical trials with scAAV9 currently use either an IT or IV delivery route, but our results support a rationale for dosing patients by both routes simultaneously. Our preliminary results in mice also suggest the possibility of treating moderately affected patients.

551. Induction of Cav1.4 in the Mature Cav1.4 Knockout Retina Rescues Synaptogenesis

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The recent FDA approval of gene therapy to treat one form of Leber congenital amaurosis has motivated intensive studies seeking to maximize the use of AAV mediated gene delivery to treat more forms of inherited retinal disease. For genes not suitable for viral packaging or aggressive degenerative diseases, the field is exploring the potential of cell replacement therapies. The assumption critical to the success of these strategies is that gene or cell replacement will activate the phenomenon of intrinsic synaptic plasticity to restore functional photoreceptor synapses. Photoreceptor synaptic development is dependent on Cav1.4, the voltage gated calcium channel clustered

at the active zone of rods and cones, just beneath the ribbon. The purpose of this study was to test if restoring Cav1.4 to the mature Cav1.4 knockout mouse photoreceptor could trigger synaptogenesis. Since the coding sequence for Cav1.4 exceeds the packaging limit of gene therapy approved vectors, we used *in vivo* electroporation to transfect mouse rod photoreceptors. Rods are electrocompetent on the day of birth (P0), but not afterwards, so electroporation of FLAG-tagged Cav1.4 or a tamoxifen inducible version of FLAG-Cav1.4 was conducted on neonates. Rod synaptogenesis is completed by P15 and the rod is fully functional by P21, so electroporated animals were treated with tamoxifen and analyzed after P21. Synaptogenesis was assessed by immunostaining for PSD-95 and RIBEYE. These two hallmark proteins are indicators of a mature functional rod ribbon synapse, and a behavioral swim test was used to determine restoration of vision. Rods electroporated with FLAG-Cav1.4 expressed PSD-95 and in half of the treated cells the RIBEYE-labeled synaptic ribbon was elongated. Treated animals had a significant improvement in the ability to pass the swim test. The inducible FLAG-Cav1.4 similarly rescued synaptogenesis, with 99% rescue of PSD-95 and 56% rescue of elongated RIBEYE-labeled synaptic ribbons. One out of four animals tested to date also passed the swim test. Intriguingly, the induced Cav1.4 only needed to be present in the terminal, not clustered beneath the ribbon to cause this effect. We conclude that the rod synapse is plastic enough to be rescued in a mature retina. To circumvent the packaging limit of AAV, we are testing intein mediated post-translational splicing to deliver Cav1.4 via two separate viruses. *In vitro* data demonstrates that the channel can be reconstituted using this method. Our central finding sets the stage for developing restorative therapies for inherited human diseases resulting specifically from a loss of Cav1.4 such as congenital stationary synaptic disease or acquired forms of retinal injury associated with down regulation of Cav1.4 such as detachment or aging. Future work will test more clinically feasible approaches for gene delivery of Cav1.4.

552. Gene Therapy for Brain Lesions in Tuberous Sclerosis Complex in Mouse Models

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Tuberous sclerosis complex (TSC) is an autosomal-dominant disorder caused by second hit somatic mutations in tumor suppressor genes, *TSC1* or *TSC2,* encoding hamartin or tuberin, respectively. These proteins act as a complex which inhibits mTOR-mediated cell growth & proliferation. Loss of either protein leads to overgrowth of cells in many organs, most commonly affecting the brain, kidneys, skin, heart & lung. We have developed stochastic mouse models of TSC brain lesions in which complete loss of Tsc1 or Tsc2 is achieved in multiple cell types in the brain by injection of an adeno-associated virus (AAV) vector, AAV1-Cre recombinase into the brain ventricles near the day of birth (P0 - P3), leading to shortened life span (mean 26-36 days), and brain pathologic findings consistent with TSC. Intravascular injection of AAV9-CBA-hamartin at day 21 in the TSC1 model led to healthy survival of over 250 days. Unfortunately, the size of the tuberin cDNA (for the TSC2 model) exceeds the packaging capacity of AAV. Here, we engineered a condensed form of tuberin (cTuberin) comprised of discrete functional domains of the parent protein, the cDNA for which fits into an AAV vector. Intravascular injection of AAV9-CBA-cTuberin in young pups in the TSC2 model extended life span by 20 days in an apparently healthy condition & approximately 55% increase in survival compared to control treated mice. We are exploring different routes of delivery & cause of death to see if we can extend the lifespan in the TSC2 model.

553. Choroid Plexus-Targeted Viral Gene Therapy with Recombinant Adeno-Associated Virus Serotype 6 (rAAV6) for Lysosomal Storage Disease

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The choroid plexuses are highly vascularized structures that project into the ventricles of the brain. The polarized epithelia of choroid plexuses produce cerebrospinal fluid by transporting water and ions into the ventricles from the blood and secrete a large number of proteins. We demonstrated that lateral ventricle administration of recombinant adeno-associated virus serotype 5 (rAAV5) resulted in selective gene transfer to the choroid plexus epithelia and rescued a mouse model of Menkes disease, a lethal pediatric disorder of copper transport. Subsequently, we assessed the feasibility of targeting choroid plexus epithelia with rAAV gene therapy vectors for treatment of a broader category of neurometabolic diseases, lysosomal storage disease (LSD). Lysosomes are compartments that function as the primary digestive units within cells and specific enzymes within lysosomes normally break down nutrients. However, patients with LSDs are unable to metabolize these nutrients, resulting in greatly diminished lifespans and reduced quality of life. There are no ideal therapeutic options currently available, especially for the CNS manifestations of LSDs. Cerebrospinal fluid-directed recombinant enzyme replacement has shown great promise for several LSDs but requires repeated intrathecal administration due to short enzyme half-lives. In contrast, rAAV-mediated gene transfer of missing lysosomal enzymes to choroid plexus epithelia enables continuous synthesis and secretion of the missing enzymes into the CSF and penetration to cerebral and cerebellar structures. CSF flow carries molecules throughout the ventricular system into the subarachnoid compartment and Virchow-Robin spaces; thus proteins present in CSF ultimately can reach the entire brain. Due to their neuroectodermal origin, choroid plexus epithelia do not turnover, rendering these cells ideal gene therapy targets. The phenomenon of metabolic cross-correction in LSDs and the immune privileged status of the brain represent other elements that augur extremely well for the success of this approach. We recently identified robust and selective transduction of choroid plexus epithelia by AAV serotype 6 (rAAV6) in a mouse model of alpha-mannosidosis, a prototypical lysosomal storage disease. This resulted in restoration of normal activity of the missing lysosomal enzyme, lysosomal alpha-mannosidase (LAMAN) in all regions of the brain, including cerebellum, considerably distant from the site of administration (lateral ventricles). The activity was sustained at least up to 6 months after a single rAAV6 injection on day 3, supporting the choroid plexus hypothesis of global brain treatment.
Mouse Cerebellum LAMAN activity (nmole/mg/hr)

	1 month	2 month	6 month
rAAV6-treated AMD Mutant	21.28 ± 4.74 n=6 p=0.0004 (Mut) p=0.1779 (WT)	17.56 ± 6.26 n=3 p=0.0031 (Mut) p=0.531 (WT)	13.87 ± 2.12 n=3 p=0.0009 (Mut) p=0.0367 (WT)
Untreated AMD Mutant	5.45 ± 0.53 n=11	4.32 ± 0.94 n=11	4.02 ± 0.43 n=5
Wild Type	29.14 ± 3.19 n=8	21.15 ± 2.28 n=8	19.78 ± 1.2 n=10

The potential impact on clinical practice in the field of LSD is high. If rAAV6-mediated viral gene therapy targeting choroid plexus epithelia is successful, the largest current barriers to health for patients with numerous different LSDs would be circumvented. In theory, more than 40 individual LSDs are amenable to this approach.

554. AAV.7m8-aflibercept Provides Long Term Protection in a Nonhuman Primate Model of Wet Macular Degeneration over One Year Post Intravitreal Vector Administration

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Exudative or wet age-related macular degeneration (wAMD) is characterized by the growth of abnormal blood vessels underneath the retina (choroidal neovascularization, CNV). Vascular endothelial growth factor (VEGF) plays a key role in in the development of CNV in wAMD and is the target of several standard-of-care therapies, including aflibercept (Eylea®). With relatively frequent intravitreal anti-VEGF injections, patients can expect to maintain and even improve visual function. However, the burden, on both the patient and the healthcare system of these anti-VEGF injections is significant. Gene therapy offers the possibility to continuously deliver anti-VEGF agents to the retina. We hypothesized that a single intravitreal administration of a vector expressing aflibercept, AAV.7m8-aflibercept (ADVM-022), could suppress pathological ocular neovascularization secondary to wAMD and alleviate the need for repeated aflibercept administration. In this study, we sought to evaluate the long-term efficacy of ADVM-022 in the laser-induced CNV model in nonhuman primates. African green monkeys received intravitreal injections of vehicle or ADVM-022 bilaterally (N=8 eyes per group, 100 µL, $2x10^{12}$vg/eye) and 13 months later, animals underwent laser photocoagulation in the macula (9 laser spots in a square grid pattern). Four additional animals received a bolus of Eylea® bilaterally (N=8 eyes, 30 µL, 1.2 mg/eye) at the time of lesioning. Clinically relevant Grade IV lesions were evaluated at 2 and 4 weeks post lesioning by fundus angiography following intravenous fluorescein administration. ADVM-022 was well tolerated with no serious long-term ophthalmic or systemic adverse events. Vehicle treated animals exhibited 42.8% and 40.3% incidence of Grade IV lesions at 2 and 4 weeks post lesioning, respectively. In contrast, the incidence of Grade IV lesions in the ADVM-022-treated eyes was 0% and 6.3%, similar to the eyes treated with aflibercept recombinant protein, which showed 2.8% and 4.5% Grade IV lesions (2 and 4 weeks post lesioning respectively in both groups). Efficacy of ADVM-022 and aflibercept in preventing the development of Grade IV lesions was highly significant ($p<0.0001$ in both groups against vehicle), however, no statistical difference was found between ADVM-022 and aflibercept groups ($p=0.4$ and $p=0.7$ at 2 and 4 weeks post laser, respectively). ADVM-022 derived aflibercept concentrations in the vitreous of un-lasered animals used as surrogates (N=6 eyes) remained stable at approximately 3 µg/mL throughout the 13-month duration of the study. In conclusion, a single intravitreal administration of ADVM-022 over a year prior to laser-induced CNV was found to be safe and highly effective at preventing the development of Grade IV lesions, similar to aflibercept administration at the time of lasering. The potential for less frequent dosing, and efficacy more than a year after a single dose, could improve compliance with anti-VEGF therapy, potentially resulting in improved efficacy overtime.

555. Selection of an AAV Gene Therapy Targeting SOD1 for the Treatment of SOD1-ALS

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Mutations in superoxide dismutase 1 (SOD1) result in progressive motor neuron loss through gain-of-function toxic properties and are responsible for up to 20% of familial ALS. Studies using transgenic mice expressing SOD1 mutations have demonstrated reduced neuropathology, improved motor behavior and extension of survival with partial lowering of SOD1. RNA interference (RNAi) is a naturally occurring process that mediates gene silencing. Expressing RNAi using artificial pri-miRNAs is the preferred approach for an AAV gene therapy targeting SOD1 for inhibition by RNAi. Here, we report a series of in vitro and in vivo studies to select an AAV gene therapy targeting SOD1 with RNAi for the treatment of ALS. To select a RNAi sequence targeting human SOD1 (hSOD1), we identified 150+ sequences that were predicted to be highly selective for hSOD1. Synthetic siRNA duplexes were first screened in Hela cells. The most effective siRNAs were then compared using 10-point dose-response curves for SOD1 mRNA suppression in Hela cells, SH-SY5Y cells, U87MG cells and primary human astrocytes. The most potent RNAi sequences were cloned into different pri-miRNA cassettes, then screened in 5 different human cell lines for SOD1 mRNA suppression. The best 12 candidates were used to generate AAV vectors for in vivo studies in transgenic mice expressing human wild-type SOD1. We employed intra-striatal dosing as a surrogate route of administration to evaluate SOD1 mRNA and protein suppression by RT-qPCR and ELISA, respectively, and to assess precision and efficiency of miRNA processing with deep sequencing. The top 2 pri-miRNA cassettes/RNAi sequences were selected for further evaluation in non-human primates by intrathecal administration of AAV vectors. SOD1 suppression was evaluated with RT-qPCR on laser captured motor neurons, while precision and

efficiency of miRNA processing was evaluated with deep sequencing on tissue samples. AAV.miR-hSOD1 significantly and safely suppressed SOD1 in lower motor neurons in non-human primates. Recently, we explored additional routes of administration in large animal models and observed broad bio-distribution and robust SOD1 lowering in the spinal cord. Evaluation of novel capsid/route of administration combinations for improved pharmacological effects of AAV.miR-hSOD1 is ongoing. Our findings support the use of AAV gene therapy targeting SOD1 with RNAi as a potential approach for the treatment of SOD1-ALS.

556. Delivery of an Allele-Specific Transcription Activator-Like Effector via AAV9 in a Transgenic Mouse Model of Huntington's Disease

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Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by the presence of a misfolded mutant *Huntingtin* (muHTT) protein. Reduction of HTT is an attractive therapeutic approach, however one must take into consideration the role of the normal, non-expanded version of *Huntingtin.* An ideal therapeutic would selectively silence only the expanded allele, affect a large population of cortical and striatal neurons, and have a durable effect. We have previously shown allele-specific silencing of the *muHTT* transcript in patient-derived fibroblasts via transcriptional activator-like effectors (TALE) by targeting a single nucleotide polymorphisms (SNP) that is highly associated with the mutant allele. Furthermore, we have demonstrated significant reduction of the muHTT (approximately 50%) and an observable reduction of the muHTT protein following unilateral striatal injection of the TALE into transgenic HD mice.In the present study, we examine the use of an adeno-associated virus (AAV) as putative delivery vehicle for our therapeutic TALE transgene in the YAC128 transgenic mouse model of HD. AAV9-TALE was directly injected into the striatum of YAC128 between 4 and 8 months of age. Mice were then tested on a motor coordination task to evaluate functional recovery. Brains were analyzed at 10-, 28-, and 90-days following injection via IHC for expression of the TALE, co-localization of the TALE with cortical and striatal neurons, co-localization in glial lineages, and for reduction of muHTT aggregates. A subset of animals was used for molecular assessment for reduction of muHTT at the RNA and protein level. TALE expression, co-localization with striatal neurons, and muHTT reduction was observed out to 90 days post-injection. An attenuation of motor deficits was also observed up to three months following injection. Identification of a potent, widespread delivery vehicle and assessment of the long-term duration of expression and effect of our therapeutic transgene will be vital in the evaluation of our TALE as a viable therapeutic for HD.

557. A Metallothionein-Based Neuroprotective Gene Therapy Approach for Lysosomal Storage Disorders

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Lysosomal Storage Disorders (LSDs) are a broad class of inherited metabolic diseases due to the defective activity of specific lysosomal enzymes. Central nervous system (CNS) manifestations are present in roughly 50% of LSDs and represent an unmet medical need for patients. Current therapies include enzyme replacement and hematopoietic cell transplantation (HCT), but for most LSDs these approaches are not effective in treating the neurologic symptoms, due to their inability to either target the central nervous system or to intervene on neurodegeneration in a timely manner. Novel experimental treatments are becoming available intended at improving CNS targeting and potentiate clinical benefit that include in vivo and ex vivo gene replacement strategies. In this study, we explored the therapeutic potential of an alternative gene therapy strategy based on the delivery of Metallothioneins (MTs), a newly identified family of proteins with reported neuroprotective roles, as a potential comprehensive approach to provide benefit to multiple LSDs affecting the nervous system and possibly other neurodegenerative conditions. This prospective study was based on the evidence that the MT gene family are over-expressed in the central nervous system and peripheral blood of patients and mice affected by LSDs, increasing during disease progression and returning to basal levels upon efficacious treatment (Cesani et al., Annals of Neurology 2014). Based on the neuroprotective functions exerted by MTs in acute and chronic brain diseases and on our findings on MT role in LSDs, we challenged the concept that an increased MT expression in the LSD brain could be associated to therapeutic effects and result in neuronal protection from degeneration. As proof of concept experiments we generated hybrid animals by crossing MT-1 overexpressing transgenic mice (MTtg) with mouse models of Batten and Krabbe diseases and assessed changes in survival and disease phenotypes of the MT-1 over-expressing disease mice. Constitutive expression of MT-1 exerted favorable phenotypic effects in both mouse models. MTtg-LSD mice survived longer and disease progression was slower than in non-transgenic LSD mice. The most profound effect was the rescue of Purkinje cells from degeneration and apoptosis observed in both models. This effect was accompanied by i) modulation of the activated inflammatory microglia phenotype with acquisition of markers of anti-inflammatory/neuro-protective cells, and ii) reduction of oxidative stress. After having obtained such promising findings in these exploratory studies, we analyzed the effect of MT-1 gene delivery to the CNS by adeno-associated virus (AAV) vectors in the same Batten and Krabbe disease models, as well as in the animal model of metachromatic leukodystrophy. Importantly, the very same effects observed in transgenic mice were obtained when MT-1 was delivered to the LSD brains by gene transfer, thus paving the way for

the development of a comprehensive gene therapy strategy targeting CNS disease in LSDs. Based on these results, we propose MTs as novel therapeutic agents (and targets) for LSDs, that could also potentiate the effect of therapeutic approaches aiming at correction of the disease-causing enzyme deficiency in the CNS and can be possibly applied to all LSDs with CNS involvement.

558. CRISPR/dCas9-Mediated Upregulation of Endogenous Neuronal-Fate Determining Genes in Somatic Cells Generates Induced Neurons for Patient-Specific Disease Modeling

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Disorders of the central nervous system (CNS) result in one of the largest economic burdens on society. While research has made enormous contributions in the identification of CNS disease-related genes, the underlying molecular mechanisms associated with disease pathology in human neuronal cells remain unclear. Developing a method to recapitulate disease- and age-associated phenotypes in human neurons grown *ex vivo* is critical for furthering understanding on CNS disorders and advancing therapeutic development. Our research uses CRISPR guide RNAs (gRNA) with a nuclease-deficient Cas9 (dCas9) fused with a transcriptional activator to upregulate endogenous "neuronal-fate" determining genes in somatic cells to facilitate transdifferentiation to induced neurons (iN). We have successfully unregulated endogenous *BRN2*, *ASCL1*, *MYT1L* and *NEUROD1* (*BAMN*) in wild-type fibroblasts following delivery of a CRISPR-dCas9 activation complex and gRNAs targeted to *BAMN*. Our time course studies show downregulation of fibroblast markers and upregulation of immature, developing and mature neuronal markers via qPCR. Immunocytochemistry revealed subpopulations of cells that are positive for neuronal markers suggesting transdifferentiation. We are currently investigating methods to increase the reprogramming efficiency through generation of inducible dCas9/VP64 cell lines and multiplex *BAMN* gRNA expression plasmids. Our future studies will study iN from Huntington's disease patient-derived fibroblasts and CDKL5-deficient patient-derived lymphoblasts, thereby enabling disease- and age-associated phenotypic analysis in patient-specific iN. To our knowledge we are the first to demonstrate dCas9/VP64-mediated upregulation of *BAMN* in human somatic cells. iN generated from patient-derived somatic cells will create an efficient disease model for investigators to study the molecular mechanisms of CNS disorders and advance the development of gene and cell therapies.

559. Vector Optimization Dramatically Improved the Outcome of Krabbe Disease Gene Therapy in the Twitcher Mouse Model

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Deficiency of galactosylceramidase (GALC) leads to Krabbe disease, a fatal lysosomal storage disease with no cure. Twitcher mice, the most commonly used animal model, have a median lifespan of 36 to 40 days. Various experimental therapies have been tested such as enzyme replacement, substrate reduction and bone marrow transplantation. But these treatments only extend the median lifespan by ≤ 40 days. Recently, adeno-associated virus (AAV)-mediated GALC gene replacement therapy was explored in neonatal twitcher mice using AAV-2, 5, 9 and rh10. However, AAV therapy alone produced limited benefits. Combining AAV and bone marrow transplantation yielded better survival than AAV alone, but the median lifespan was only extended by ~80 days in the combination therapy. To improve the efficacy of AAV gene therapy, we engineered an optimized AAV-9 GALC vector to enhance the production, secretion and blood-brain-barrier crossing of AAV produced GALC. A single intravenous injection of the optimized vector in newborn twitcher mice resulted in high efficient gene transfer, significant improvement in body weight and motor function. Most importantly, the median lifespan of the treated mice reached 150 days (range 112 to 180 days). This greatly exceeded all published data. Our results suggest that systemic AAV-9 delivery of the optimized vector is a highly promising approach to treat Krabbe disease (Supported by the Kansas City Area Life Sciences Institute - Patton Trust Research Grant and NIH).

560. An Innovative Combinatorial Hematopoietic Stem Cell Transplantation Strategy Enhances the Therapeutic Potential of HSC-Based Strategies in Mucopolysaccharidosis Type II

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Mucopolysaccharidosis type II (MPS II), also known as Hunter syndrome, is an X-linked inherited lysosomal storage disorder (LSD) that affects approximately 1 in 100,000 to 1 in 170,000 males. It is due to deficiency of the iduronate sulfatase (IDS) enzyme that results in the accumulation of the glycosaminoglycans (GAGs) in many tissues including the central nervous system (CNS). Available treatments such as enzyme replacement therapy and hematopoietic cell transplantation

(HCT) mostly alleviate somatic and non-neurological symptoms, and are poorly effective on brain disease manifestations. The inability of the defective enzyme to efficiently cross the blood-brain barrier (BBB) and the slow pace of replacement of resident CNS myeloid/microglia populations with metabolically competent donor derived cells accounts for the limited benefit of the available treatments. To anticipate and enhance the transplant contribution to brain myeloid cells, and consequently increase and foster IDS enzyme delivery to the MPS II CNS and clinical benefit of HCT, we investigated a newly developed transplant approach based on the direct delivery of donor/gene corrected autologous hematopoietic stem/progenitor cells (HSCs) in the lateral ventricles of the brain (intracerebral ventricular - ICV - delivery)(Capotondo, Milazzo et al., Sci. Advances 2017). We transplanted wild type (WT) donor HSCs or IDS-/- control HSCs into busulfan myeloablated MPS II recipients, comparing a standard intra-venous (IV) cell delivery approach to a combined IV+ICV transplantation strategy. Six months after the transplant, treated and control animals were tested by behavioral studies. Interestingly, both the rotarod test and the Y maze test showed a better performance of mice receiving a combined ICV+IV cell transplant as compared to the other groups, indicating that the administration of an additional dose of the WT HSCs administered in the brain ventricles could enhance the therapeutic efficacy of the transplantation on neurologic disease abnormalities. Consistently with these in vivo findings, we observed a higher donor cell chimerism within myeloid brain populations in MPS II mice receiving the donor HSCs IV+ICV as compared to the control IV-only group. Enzyme activity and GAGs quantification on the brain tissue is currently on going and could provide additional support to our experimental hypothesis. Based on these promising data, we then tested whether the same additive therapeutic effect could also be observed in a HSC gene therapy setting. We thus transplanted IDS-/- HSCs transduced with a lentiviral vector encoding IDS or with a reporter vector by IV-only delivery or by the combined IV+ICV approach in busulfan treated MPS II young recipients and began a clinical follow up. Interestingly, preliminary results from mid-term evaluation of some of the treated animals indicate a treatment associated effect and may suggest that the combined transduced cell delivery could be particularly beneficial. The long-term follow up of the entire on-going experimental cohorts will allow confirming these preliminary indications as well as assessing the safety of this approach. A research grant from Orchard Therapeutics contributed to the financial support of this work.

561. Homology Independent Targeted Integration for Gene Correction in Photoreceptors

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Retinitis pigmentosa (RP) is a heterogeneous group of inherited ocular diseases affecting /3.000-5.000 people worldwide. Thirty-40% of all cases of RP have an autosomal dominant inheritance. Mutations in the rhodopsin gene (*RHO*) are responsibble for about 20-25% of cases of dominant RP. Genome editing for dominantly inherited diseases should knock out the mutant allele leaving the wildtype unaffected. Alternatively, gene correction by homologous recombination can be used, which is however inefficient in neurons. To overcome these challenges, Homology-Independent Targeted Integration (HITI) has been developed[1], which allows precise integration of a donor DNA template at a site of interes, by non-homologous end joining of the DNA template within the genomic locus, after both have been cleaved by CRISPR/Cas9. To adapt this system for mutation-independent RHO correction, we have designed a guide RNA (gRNA) specific for the first exon of murine RHO (mRHO), and a donor DNA template carrying a STOP codon - to knock out the expression of the endogenous mRho gene -, a translation inititation sequence and either the reporter gene dsRED or RHO. The donor DNA is flanked by the same mRho gRNA cleavage sites. We have tested this system in vitro and found that 77,6% of transfected cells were dsRED+ when the mRHO- but not a scrabmle gRNA was used. To test HIYI in the mouse retina, we generated one adeno-associated viral (AAV) vector that expresses SpCas9 under the control of a photoreceptor-specific promoter, and one that carries the HITI donor template, the mRho (or a scramble) gRNA expression casette and GFP under control of a retinal pigmented epithelium-specific promoter. Subretinal injection of 2.5*10^9 genome copies of each vector in C57BL/6 mice resulted in dsRED signal detected in 6-7% of total rods in the transduced region at forty days after injection when mRho but not the scramble gRNAs were used. Experiments to integrate a correct copy of RHO in photoreceptors of a mouse model of dominant RP are in progress with the goal of developing a RHO mutation-independent therapeutic approach for dominant RP.

1. Suzuki et al, Nature. 2016 Dec 1;540(7631):144-149

562. Identification of Synuclein-γ (SNGC) Promoter as a Novel Retinal Ganglion Cell-Specific Promoter for Neuroprotection Gene Therapy

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The loss of retinal ganglion cells (RGCs) is the hallmark of optic neuropathies, including traumatic optic nerve injury, glaucoma and optic neuritis. Gene therapy for neuroprotection holds great promise in rescuing disease associated-RGCs. Adeno-associated virus (AAV) has served as a primary vehicle for gene therapy and provided effective treatment for inherited retinal diseases, due to its minimal immune responses and sustained transgene expression. AAV2 preferentially infects RGCs in the ganglion cell layer (GCL) after intravitreal injection, however, it also infects cells in other layers of retina, which limits its use for RGC-specific gene targeting. We reasoned that a RGC-specific promoter may overcome this hurdle, but unfortunately there is no an experimentally proved RGC-specific promoter available. Here, we screened six mouse promoters that have been indicated in RGC expression before (synuclein gamma[mSncg], a-calcium/calmodulin- dependent protein kinase II[mCaMKIIa], ISL LIM Homeobox 2 [mIls2], thy-1 cell surface antige[mThy1], Class III Beta-Tubulin[mTuBB3], and synapsin I [mSynI]) and compared with the ubiquitous CMV early enhancer/chicken β-actin (CAG) promoter. Using the same AAV vector backbone, we replace the CAG promoter with the tested promoters to drive the expression of enhanced green fluorescent protein (EGFP). We infected AAVs with primary human

retina cells freshly isolated from human donor eyes. The mSncg-EGFP showed a strong and specific expression in human RGCs evidenced by high co-localization ratio with RBPMS, a specific RGC marker. On the contrary, most mThy1-EGFP positive cells are not RBPMS positive. For in vivo studies, we injected AAVs intravitreally into mouse eyes at the dose of 3.0×10^9 vg/eye. The strength of mSncg promoter or mCaMKIIa promoter is similar with CAG promoter in mouse RGCs in vivo, followed by mIsl2, and mThy1, mTuBB3 and mSyn1 promoters, which showed lower EGFP expression in RGCs by retina wholemount staining. By cross section of eyeballs, we can detect the specificity of these promoters in different layers of retina. Among the promoters that we tested, mSncg and mThy1 promoter showed preferential EGFP expression in GCL, in dramatic contrast to CAG promoter, EGFP of which was not only in GCL, but also throughout the inner nuclear layer. Since we found that mSncg promoter has much stronger activity than human Sncg promoter in mouse eyes, we further characterized the mSncg promoter and determined the optimal size and critical region for its specificity and activity. Finally we demonstrated RGC-specific gene targeting using AAV2-mSncg-Cre in *eIF2α A/A;fTg* transgene mice, indicating the potential application of this promoter in combination with AAV2 for RGC gene therapy.

563. AAV Gene Therapy for Inherited Childhood Epilepsy, Dravet Syndrome

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Dravet syndrome is an incurable, inherited childhood epilepsy caused by a mutation in the SCN1A gene, which encodes the voltage-gated sodium channel NaV1.1. Dravet Syndrome has an incidence rate of 1:20,000 to 40,000 live births and patients suffer from fever-sensitive, refractory and generalized seizures and cognitive impairment, which begin at around six months of age. Patients can fall into status epilepticus, which can result in premature death. This disease remains untreatable by either medical or surgical means. SCN1A knock-out mice display an epileptic phenotype similar to human Dravet patients and die by day 12. Gene therapy for Dravet syndrome faces several challenges. Firstly, we and others have experienced difficulties in propagating wild-type SCN1A plasmids in competent cells (Feldman and Lossin, 2014). Secondly, the large transgene limits incorporation into AAV gene therapy vectors. Therefore, we have designed a bipartite AAV system, in which SCN1A is split into two complementary halves, one of which has been codon-optimized, based on a previous study (Stühmer et al., 1989). Within these two AAV plasmids we incorporated a human synapsin promoter which drives each half of the gene (called AAV-A and AAV-B). In addition to this, we also incorporated reporter genes RFP and GFP linked by a bicistronic linker to the two plasmids; AAV-A-RFP and AAV-B-GFP. To test whether the two AAV plasmids would co-express and produce a functional sodium ion channel, we have transiently transfected the plasmids into N2a neuronal cells and subsequently patch clamped cells which expressed both GFP and RFP. The electrophysiology results revealed that co-transfecting the two halves produced functional sodium channels. Recordings showed voltage dependent sodium currents, which were not seen in the controls (Figure 1, n=3). This is a novel approach and is the first use of AAV to restore full-length SCN1A coding sequence in neuronal cells.In conclusion, we have shown that the two halves of the SCN1A gene do form a functional protein when co-expressed from two different plasmids. Future development of this novel gene therapy for Dravet patients would involve testing the approach in the Dravet mouse model and monitoring whether this ameliorates the disease phenotype.

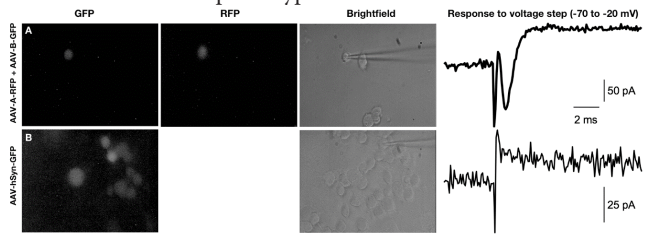

Figure 1. AAV-A-RFP and AAV-B-GFP produces a functional Nav1.1 channel detected after current injection (A). Cell transfected with a control plasmid only encoding GFP protein show no response (B)

564. Biochemical Differences between Human and Mouse Lysosomal GLB1 and the Role of Protective Protein/Cathepsin A in Protein Stabilization

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Background: GM1 gangliosidosis is a lysosomal storage disease caused by mutations in the *GLB1* gene encoding the lysosomal hydrolase acid beta-galactosidase (βgal). βgal deficiency causes the toxic accumulation of GM1 ganglioside in neurons. Previously we have shown that treatment of *Glb1*$^{-/-}$ mice by IV delivery of an AAV9 vector encoding mouse βgal increased total βgal activity in brain and spinal cord and prolonged survival significantly. However, subsequent comparative studies in C57BL6/J mice showed that βgal activity was 10-fold lower in mice treated with AAV9 encoding the human protein when compared to the mouse. βgal resides in the lysosome as a large megadalton multiprotein complex that includes, among others, neuraminidase (Neu) and Cathepsin A, also known as protective protein/cathepsin A (PPCA). In humans, mutations in the *CTSA* gene encoding PPCA cause galactosialidosis, a combined deficiency of βgal and Neu, due to rapid degradation in the lysosome or a lysosomal transport deficit, respectively. Interestingly *Ctsa* knock-out mice display reduced Neu activity similar to galactosialidosis patients, but normal βgal activity. This led us to hypothesize that human β-galactosidase enzyme is less stable than mouse β-galactosidase without the benefit of a species-specific protective protein/Cathepsin A. Methods: Human or mouse AAV9-βgal vectors (1E12 vg/mouse) were co-injected systemically with human or mouse AAV9-PPCA vectors (1E12 vg/mouse) in 6 week-old C57BL/6J mice (n=5/group). Control groups included treatment with each AAV9 vector alone, and PBS. Tissues were harvested 3 weeks post-injection and the following parameters assessed: βgal

and PPCA activity using fluorometric or colorimetric assays with artificial substrates, western blot analysis of βgal and PPCA expression, immunoprecipitation to assess βgal-PPCA protein interaction, and AAV biodistribution. Implications: We anticipate a significant increase in βgal activity in tissues of mice treated simultaneously with human βgal and PPCA AAV9 vectors compared to human AAV9-βgal alone, or the combination of human βgal with mouse PPCA vector. This result would indicate that the interaction of human βgal with PPCA is species specific and critical to achieve high level expression in transduced cells. Based on the observation that *Ctsa* knock-out mice show no βgal activity deficit, we do not anticipate co-infusion of mouse βgal and PPCA vectors to result in a significant increase in activity over treatment with the mouse βgal vector alone. We expect immunoprecipitation to show a species specific interaction between human βgal and PPCA. This study may reveal an important avenue to increase the efficacy of AAV gene therapy for GM1 gangliosidosis by co-expression of βgal and PPCA. In addition, it also highlights the translational challenge of testing AAV vectors encoding human proteins in animals as biochemical differences can impact efficacy and safety readouts.

565. A P23H RHO-Specific Meganuclease Rescues Photoreceptor Morphology and Function in Mouse Models of Retinitis Pigmentosa

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Autosomal dominant genetic diseases are not amenable to conventional gene replacement strategies due to the need to eliminate a gain-of-function mutation. Alternatively, genome editing approaches for these disorders offer the potential for selective elimination of the causative mutant allele while leaving the wild-type allele intact. In the US population, autosomal dominant retinitis pigmentosa (adRP) results most frequently from a C-to-A nucleotide transversion in the rhodopsin (RHO) gene, leading to substitution of proline 23 with histidine (P23H). We report the development and preclinical characterization of an engineered meganuclease that targets the P23H point mutation and selectively knocks-out the mutant RHO allele in vitro and in vivo. The nuclease efficiently edits the P23H allele in cultured cell lines, but exhibits little to no activity toward wild-type RHO in cultured cells or transduced human retina explants. Subretinal injection of an AAV5 vector encoding the meganuclease under the control of a photoreceptor-specific promoter enhanced the survival of both rod and cone photoreceptors and reduced retinal stress in $hRHO^{P23H}$ transgenic mice. Off-target analysis in cultured human cells and retina explants as well as in vivo functional rescue studies will be discussed. Collectively, these studies illustrate the potential of meganuclease-based genome editing to treat adRP and other autosomal dominant disorders.

566. A miRNA-Based Gene Therapy Approach to Target Mutated SOD1 in Key Cell Types in Amyotrophic Lateral Sclerosis {ALS}

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Background: Amyotrophic Lateral Sclerosis {ALS} is a neurodegenerative disease characterized by progressive death of motor neurons {MNs} leading to fatal paralysis. No cure currently exists for this devastating disease. ALS cases are grouped into two categories: sporadic ALS {sALS} and familial ALS {fALS}. 20% of the latter are caused by mutations in the superoxide dismutase 1 {SOD1} gene. Interestingly, SOD1 mutations are also pervasive in sALS. Therefore, one sound approach for the treatment of SOD1-related ALS is to downregulate the expression of mutated SOD1 in cells that are vulnerable to the disease using RNA interference {RNAi} as a potential gene therapy for fALS and sALS. We have developed a cell-specific bicistronic adeno-associated viral {AAV} vector cassette that can selectively express an artificial murine-miRNA {mmu-miRhSOD1} in MNs {hSyn promoter} and astrocytes {$gfaABCD_1D$ promoter}. We found that when administered into the cerebrospinal fluid {CSF} of SOD1G93A ALS mice, AAV9 can effectively target MN and astrocytes in the spinal cord and allows for the continuous expression of artificial miRNAs against hSOD1. **Preliminary results:** Juvenile mice aged 5 weeks were injected intrathecally with the bicistronic AAV9 mmu-miRhSOD1. MN survival, neuromuscular junction occupancy, electromyography and behavioral tests were carried out to assess efficacy of our vector on disease progression. Results confirmed the synergistic effects of expressing the artificial mmu-miRhSOD1 in both neurons and astrocytes, as compared to targeting either solely MNs or solely astrocytes. Analysis of the animals at 140 days of age revealed increased survival of MNs at the lumbar level and dramatically enhanced occupancy of the neuromuscular junctions in the gastrocnemius muscle. Additionally, we observed enhanced preservation of the compound muscle action potential in the triceps surae as well as a boost in the swimming performance towards disease end stage. **Rationale:** The key question is whether this approach can be optimized for application in ALS patients. **Objective:** Design an artificial human-miRNAs targeting hSOD1 {hu-miRhSOD1} that could be expressed via our bicistronic vector for translation of the gene therapy in fALS patients. **Methods:** 7 artificial human-based miRNAs were designed and their efficiency tested in HEK293T. Processing accuracy was assessed using next-generation sequencing. **Results:** The silencing potential of the hu-miRhSOD1 sequences was tested in vitro and demonstrated equal or superior efficiency compared to the original mmu-miRhSOD1. Evaluation of miRNA processing, showed that 99% of the hu-miRhSOD1 produced contained the predicted seed sequence and 5 out of 7 hu-miRhSOD1 showed minimal expression of the complementary passenger strand, therefore limiting the risk of potential off-target effects. **Conclusions:** Given the robust rescue of motor function obtained following our gene therapy approach, the promising bicistronic AA9 vector expressing the newly designed humanized miRhSOD1 should be evaluated in a translational context for implementation in fALS patients. Acknowledgements: Wyss Center, Campus Biotech, Geneva, Switzerland.

567. Using Patient Specific iPSCs to Evaluate the Pathophysiology of Novel WFS1 Mutations

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Purpose: Mutations in Wolframin ER transmembrane glycoprotein 1 (WFS1) gene have been associated with optic atrophy (OA) and Wolframin syndrome. The purpose of this study was to use patient specific iPSCs to determine how novel mutations in *WFS1* cause isolated retinal ganglion cell (RGC) death. **Methods**: Dermal fibroblasts were obtained and expanded from two patients with suspected *WFS1* associated optic atrophy and one patient with molecularly confirmed Wolframin syndrome. Fibroblasts were targeted for induced pluripotent stem cell (iPSC) generation using Sendai viruses driving expression of OCT4, SOX2, KLF4 and c-MYC. Pluripotency was confirmed using rt-PCR, immunocytochemistry and the TaqMan Scorecard Assay. Patient derived iPSCs were differentiated into RGCs and b-islet cells. Immunofluorescence, TaqMan ER stress expression and Western blot analyses were used to characterize expression of ER stress associated markers. Cells generated from normal non-diseased controls and a patient with molecularly confirmed Wolframin syndrome were used as controls. **Results:** Homozygous Arg558Cys WFS1 variants were identified in two patients with non-syndromic recessive optic atrophy via exome sequencing. As determined by a TaqMan ER stress assay, patient-specific iPSC-derived retinal ganglion cells generated from these two individuals were found to have increased levels of key ER stress genes such as *BIP* and ERO1LB. Likewise, ER-stress mediated RGC dysfunction, as evident by increased levels of *BIP*, HSP90AB, *CANX*, *DDIT3*, *EIF2AK3*, *CASP3* and *BAX* were identified. Interestingly, unlike the patient with Wolframin syndrome, no evidence of ER-stress was detected in b-islet cells generated from either of the patients with non-syndromic disease. **Conclusions**: Mutations in *WFS1* can cause non-syndromic early onset optic atrophy. By using patient specific iPSCs we have successfully demonstrated that mutations in *WFS1* lead to activation of the ER-stress pathway.

568. Intracranial Delivery of Re-Designed AAVrh8 Vector Expressing Hexα/β Subunits is Effective in Treating *Hexb*$^{-/-}$ Mice

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Background: Tay Sachs disease (TSD) is a fatal lysosomal storage disorder caused by mutations in the *HEXA* gene that reduce or eliminate the activity of the lysosomal hydrolase β-N-hexosaminidase A (HexA), which is responsible for catabolism of GM2 ganglioside and other metabolites. HexA is a heterodimeric isozyme comprised of α and β-subunit encoded by the *HEXA* and *HEXB* genes, respectively. Loss of HexA activity leads to accumulation of GM2 gangliosidose and ultimately neurodegeneration. AAV gene therapy is the most promising approach for treatment of TSD. Previously we have shown therapeutic efficacy of bilateral thalamic and ICV delivery in mouse, feline, and sheep models of GM2 gangliosidoses using a 1:1 formulation of two monocistronic AAVrh8 vectors encoding HexA α and β-subunit. However, the same AAVrh8 vector formulation showed marked toxicity in non-human primates. We postulated that this unexpected event was caused by excessive transgene expression in neurons and embarked on an extensive vector re-design to moderate expression levels. The following study assessed the efficacy of re-designed monocistronic AAV vectors in Sandhoff mice (SD), which also accumulate GM2 ganglioside in the brain due to knockout for the *HexB* gene. Methods: AAVrh8-CB-CI-mHexα/β were co-injected bilaterally into the thalamus and one cerebral lateral ventricle (ICV) of 4-week old SD mice. SD mice, n=15/group, were injected with 1.76E10 vector genomes (vg), 3.51E10 vg, 7.02E10 vg, or vehicle at 30 days of age. Untreated wildtype littermates (n=15) and untreated SD mice (n=15) were used as controls. Mice were sacrificed at either 60 days (n=5/group) or 150 days of age (n=10/group). Treatment efficacy was assessed using rotarod and inverted screen testing, as well as survival. The ability of the vector to produce enzyme and reduce GM2 ganglioside content was assessed biochemically using enzymatic assays and mass spectrometry, respectively. Results: The survival distributions for the six groups were significantly different ($X^2(5)=62.006$, $p<0.0001$). Untreated SD mice reached the humane endpoint at a median of 131 days. All animals in the untreated wildtype and mice injected with 7.02E10 vg reached the experimental endpoint of 150 days. During rotarod testing at 120 days, vehicle-treated and untreated SD mice had an average latency to fall of 5.3 ± 4.5 (mean ± SD) and 9.2 ± 5.29 seconds, respectively. This is in contrast to wildtype untreated mice and mice treated with 7.02E10 vg which had an average latency to fall of 235.07 ± 48.56 seconds and 202 ± 77.38 seconds, respectively. The brain was divided into 6 coronal blocks in order to characterize enzymatic distribution and GM2 ganglioside content throughout the brain. There was a significant effect of dose on HexA activity in the brain section containing the thalamus, with AAV-treated mice showing higher enzymatic activity ($p<0.0001$). There was also a significant effect of dose on GM2 ganglioside content in this brain section, with treated mice having less GM2 gangliosides in the brain ($p<0.0001$). Conclusion: Treatment of SD mice with the new generation of AAVrh8 vectors increased survival and prevented the loss of hindlimb strength and motor coordination observed in untreated animals. This was accompanied by an increase in Hex activity and a decrease in GM2 ganglioside content in the CNS. These results indicate that the re-designed AAV vectors are efficacious and safe for intracranial gene therapy of GM2 gangliosidoses.

569. Gene Therapy Correction of Frataxin Deficiency in a Friedriech's Ataxia Mouse Model

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Friedriech's ataxia (FRDA) is an autosomal recessive disorder caused by mutation in the frataxin (*FXN*) gene (intronic expansion of GAA triplets). Deficiency of FXN protein results in inefficient mitochondrial electron transfer, iron accumulation, and oxidative damage. It affects 1 in 50,000 individuals in the US. In the nervous system, FRDA primarily affects the cerebellum, spinal cord, and dorsal root ganglion leading to significant ataxia, muscle weakness, hyporeflexia, and dysmetria. Severe hypertrophic cardiomyopathy and arrhythmia are the cause of premature death in FRDA. Currently, no effective treatment exists for FRDA disease progression. The overall objective of this study is to determine the *in vivo* correction of FXN deficiency achieved by delivery of an AAV9 vector expressing human *FXN* gene (rAAV9-CBA-hFXN). We used a novel FRDA mouse model in which the endogenous mouse *FXN* gene was ubiquitously silenced via doxycycline induced shRNA knockdown. The experimental animals were treated with intra-venous (IV) and intra-thecal (IT) administration of rAAV9-CBA-hFXN to augment the level of cellular FXN and prevent the consequences of FXN deficiency in the heart and nervous system. We used functional, behavioral, physiological and imaging outcome measures to characterize the cardiac and neurologic disease phenotype and the correction achieved by hFXN restoration. The cardiac outcomes included EKG and MRI. The neurologic outcomes include grip strength, open field, rotarod, sensory evoked potential, gene expression and MRI changes. Preliminary results from the ongoing study suggest a dose sensitive preservation of cardiac and neurological function, and prevention of weight loss and death in mice treated with IV as well as IT vector administration. Completion of this project will be an important milestone in the development of a treatment strategy that will dramatically improve the quality of life for patients with Friedreich's ataxia.

570. Intravitreal Administration of an AAV6 Triple Mutant Vector Fails to Transduce Retinal Microglia

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Recent interest in the role of microglia in neurodegenerative diseases has spurred the development of sophisticated transgenic mouse lines and targeted knock-ins for in vivo manipulation of this cell type. Such tools have permitted the characterization of microglial functions and have simultaneously implicated microglial activation in numerous retinal degenerative models. Indeed, genetic ablation of microglia by specific expression of diphtheria toxin A in Cx3Cr1-expressing cells, has demonstrated a significant delay of photoreceptor degeneration in the rd10 mouse model. Unfortunately, much less success has been achieved with regards to the development of a viral vector capable of transducing microglia across a wide range of genetic backgrounds. Such a tool would greatly facilitate functional studies. Recently, specific, but modestly efficient, transduction has been reported by intracerebroventricular injection of neonatal mice using an AAV6 triple mutant capsid (Y731F/Y705F/T492V; AAV6TM) carrying a self-complementary transgene cassette driven by a CD68 promoter. Importantly, capsid modifications such as tyrosine to phenylalanine and threonine to valine have been shown to improve the ability of other AAV serotypes to transduce the retina via intravitreal administration. In order to test the ability of the AAV6TM vector to transduce retinal microglia, we packaged a self-complementary genome containing a GFP-reporter driven by a CMV promoter, and intravitreally administered a dose of 1e9 vg to the right eye of 10-week old C57BL/6 mice. The left eye of each mouse was injected with a vector excipient. Fifteen days post-injection, we performed in vivo fluorescence fundoscopy. Robust GFP expression following a roughly perivascular pattern confirmed the viability of the intravitreally administered virus. We then prepared retinal flat mounts for immunofluorescent staining of GFP and Iba1 to see if any of the transduced cells were indeed microglia. Using confocal microscopy, we observed that many of the GFP+ cells had large dendritic arbors and long axons projecting to the optic nerve head, likely representing ganglion cells. Unfortunately, we did not observe any instances of GFP and Iba1 co-localization, suggesting that the AAV6TM vector was not capable of efficiently transducing retinal microglia via the intravitreal route. Alternatively, retinal microglia may be inherently different from brain microglia, thus requiring different tropic properties. Nevertheless, since AAV6TM is capable of (1) transducing the neural retina via the intravitreal route, and (2) transducing brain microglia via intracerebroventricular administration, we conclude that AAV6TM may be a good starting point for directed evolution of a novel vector capable of transducing retinal microglia. The development of such a vector would be a valuable tool to the research community and facilitate the therapeutic targeting of microglia in retinal degenerative models.

571. CRISPR/Cas9-Mediated Gene Editing for Huntington's Disease

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Neurodegenerative disorders are a major public health problem because of the high frequency of these devastating diseases in the population. Genome editing with the CRISPR/Cas9 system is making it possible to modify the sequence of genes linked to these diseases in the adult brain. Here, a self-inactivating CRISPR/Cas9 system, kamiCas9, was designed for transient expression of the Cas9 protein and high editing efficiency. In the first application of this technology to neurodegenerative disorders, the gene responsible for Huntington's disease (HD) was targeted in adult mouse neuronal and glial cells. Mutant and wild-type (WT) huntingtin (HTT) was efficiently inactivated in mouse models of HD, leading to an improvement in key markers of the disease. Sequencing of potential off-targets with the constitutive Cas9 system in differentiated human iPS cells, revealed a very low incidence with only one site above background level. Importantly, the off-target frequency was drastically reduced with the kamiCas9 system. Based on these proof-of-principle, we now designed more complex strategies for a selective editing of mutant HTT, which represents the safest approach preserving WT HTT expression and functions. We designed sgRNA discriminating mutant and WT HTT alleles by using sequences containing Single Nucleotide Polymorphism (SNP) in the HTT gene. The editing efficiency and selectivity of these strategies were evaluated in human embryonic kidney 293T (HEK-293T) cells. We are currently evaluating the best candidates in neuronal cultures derived from HD patients.

572. CRISPR/Cas9 Technology-Based Genomic Editing in Human Pluripotent Stem Cells to Model Bietti's Crystalline Retinal Dystrophy

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Bietti's crystalline retinal dystrophy (BCD) is an inherited disease caused by mutations of CYP4V2 gene, leading to death of retinal pigmented epithelial cells (RPE) and ultimately the loss of vision. BCD-caused RPE damage and blindness is incurable currently but the pathogenesis is little understood. Hence it is a compelling need to establish an ideal disease model for exploring the pathological mechanism of BCD, and exploit new therapies for further improving clinical outcomes. Here, we generated BCD patient-specific induced pluripotent stem cells (psiPSCs) using episomal vectors and successfully knocked out CYP4V2 gene in isogenic human pluripotent stem cells (PSCs) with CRISPR/Cas9 technologies. The lipid accumulation was observed in all CYP4V2 mutant PSCs, which did not affect their viability and cell differentiation ability in vitro. However, when cells were differentiated into functional RPE cells, we eventually observed the chronic death of all these CYP4V2 mutant RPE cells during in vitro culture apart from the lipid accumulations after the cell polarization. Lipid accumulation-provoked mitochondrial stress was evidenced in BCD RPE cells. Lipid profiling revealed a panel of unsaturated fatty acids plays a critical role to increase mitochondrial oxidative stress-induced apoptosis in CYP4V2 mutant RPE cells. Replenishment of CYP4V2 expression in $CYP4V2^{-/-}$ RPE cells remarkably recovers functions and cell survival status.. We conclude CYP4V2 mutation-provoked RPE damage in human is associated with lipid accumulation-triggered mitochondrial stress and apoptosis. Gene therapy targeting CYP4V2 or medication attenuating mitochondrial stress will be considered as potential therapeutic strategies

573. Protease Inhibitors Overexpression Eliminate the Toxicity Effect on ERG In Vivo by AAV-Mediated Gene Delivery and Protect ARPE19 Cells from Oxidative Stress and Cell Death In Vitro

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Purpose Ciliary neurotrophic factor (CNTF) has been demonstrated to be effective at rescuing rod- and cone-photoreceptors in animal models of RP, but at the cost of suppressing electrophysiological responses. We previously conducted a transcriptome analysis of 23,365 mouse genes in order to determine the mechanisms underlying long-term neuroprotection of cone photoreceptors following recombinant adeno-associated virus (rAAV)-mediated over-expression of CNTF. Of the 460 genes found to be significantly differentially expressed in high dose rAAV.CNTF treated eyes comparedreated controls, peptidase inhibitor gene families were identified as being most highly over-expressed (up to 90-fold). As peptidase inhibitors are known to be anti-apoptotic, protective against oxidative stress, and to prevent degradation of the cell membrane and extracellular matrix, we sought to investigate whether they can directly protect against cone photoreceptor degeneration without ERG suppression. **Methods** Wild-type (C57Bl/6j) mice received a unilateral intravitreal injection of rAAV2/2 vector expressing either CNTF, Serpina5, Serpina3k, Wfdc6a or Timp3; the contralateral eye received a sham buffer injection of equal volume (2µl). All animals were followed up by repetitive electroretinography (ERG) to determine whether over-expression of proteolysis inhibitors has a similar suppressive effect as CNTF. A novel in vitro co-culture assay was used to determine the anti-oxidative and anti-apoptotic effects of CNTF (positive control) and each protease inhibitor. HEK293T cell monolayers were transfected with plasmid DNA expressing either CNTF, Serpina3K, Serpina5, Timp3 or Wfdc6a. ARPE19 cells were then plated in tissue culture inserts and suspended above the transfected HEK293T cell monolayer, allowing for diffusion of secreted factors (CNTF or inhibitors) from HEK293T cell monolayer to interact with the APRE19 cells. Oxidative stress and cell survival assays were performed on ARPE19 cells after incubation with 1mM H_2O_2 for one hour. A CellROX staining assay was used to detect oxidative stress levels while a calcein/ethidium bormide staining assay was used to measure cell viability. Fluorescence signals were quantified by plate reader and normalized to Hoechst staining, to control for cell number variability. **Results** 1) Four weeks post injection, ERG revealed that AAV2/2-meadiated over-expression of CNTF significantly reduced the mean amplitude of both a- wave and b- wave in dark- and light-adapted conditions. By contrast, AAV-mediated over-expression of

protease inhibitors did not affect either dark- or light-adapted ERG responses in any group. Our in vitro co-culture assay revealed that CNTF and protease inhibitors were all protective against oxidative stress compared to untranfected H_2O_2 only controls. Encouragingly, the live/dead assay demonstrated significantly more living cells and fewer dead cells in all groups relative to control. Among those, CNTF and Serpina5 significantly increased cell survival, while CNTF, Serpina5 and Serpina3k significantly prevented cell death. **Conclusions**: The preliminary data presented herein demonstrate that 1) over-expression of proteolysis inhibitors does not negatively affect ERG function in vivo compared to CNTF, and 2) that proteolysis inhibitors are protective against oxidative stress and cell death in vitro. Based on this work, we are currently evaluating the long-term rAAV-medaited protective effects of proteolysis inhibitor overexpression using a well establish mouse model of retinitis pigmentosa.

575. Long-Term Improvement of Neurological Signs and Metabolic Dysfunction in a Mouse Model of Krabbe's Disease after Global AAV9-GALC Gene Therapy

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We report a global AAV9-based gene therapy protocol to deliver therapeutic galactosylceramidase (GALC), a lysosomal enzyme that is deficient in Krabbe's disease. When globally administered via intrathecal, intracranial, and intravenous injections to newborn mice affected with GALC deficiency (twitcher mice), this approach largely surpassed prior published benchmarks of survival and metabolic correction, showing long term protection of demyelination, neuroinflammation, and motor function. Bone-marrow transplantation, performed in this protocol without immunosuppressive pre-conditioning added minimal benefits to the AAV9-gene therapy. Contrasting with other proposed pre-clinical therapies, these results demonstrate that achieving near complete correction of GALC's metabolic deficiencies across the entire nervous system via gene therapy can have a significant improvement to behavioral deficits, pathophysiological changes, and survival. These results are an important consideration for determining the safest and most effective manner for adapting gene therapy to treat this leukodystrophy in the clinic. This work was partially funded through grants from the NIH to MSM (F30NS090684) and ERB (R01 NS065808, the Legacy of Angels Foundation, and the European Leukodystrophy Association)

576. Characterization of a Novel Rat Model for HIV Neuropathogenesis

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For the past 30 years, the Human Immunodeficiency Virus (HIV) has remained a prevalent and elusive disease around the world. HIV infects the host's immune cells and weakens the immune system. This leads to increased vulnerability to insults and if untreated, can progress to acquired immune deficiency syndrome (AIDS), a disease that affects over 1.2 million people in the U.S. alone. In the central nervous system (CNS), HIV can infect astrocytes and microglia cells, leading to the development of neurodegenerative diseases such as HIV-associated neurocognitive disorder (HAND). While there are treatments available for HIV, namely highly active antiviral retro therapy (HAART), they are largely ineffective when trying to eradicate HIV infection of cells in the CNS. To better study HAND and similar diseases, we have developed a novel transgenic rat model of HIV infection, termed "iHIV". The iHIV transgenic rat contains a replication defective HIV provirus targeted to the Rosa26 locus. In this modified HIV provirus, the gag and pol genes have been replaced with a transcription termination signal flanked by loxP sites and a nanoluciferase reporter. The virus contains 5' and 3' LTRs and HIV proviral proteins. The nanoluciferase reporter gene was used to replace the gag/pol genes. These components allow for a Cre-inducible provirus, which expresses the nanoluciferase reporter and the accessory proteins under the control of the LTR promoter. Genotyping analysis confirms copy number and appropriate targeting of the iHIV construct. Injection of AAV-Cre virus into iHIV animals shows an increase in detectable Nanoluciferase reporter activity. To activate the iHIV provirus in microglia, we have developed a CX3CR1-driven CreERT2 transgenic rat to promote activation of the provirus in microglia, monocytes, dendritic cells and natural killer cells. This CX3CR1-CreERT2 rat was crossed with a rat containing a DIO-mCherry construct, and double-positive progeny were assessed for CreERT2 activity in microglia by colocalizing mCherry and the microglial marker, Iba1. These two rats have been crossed to develop a model for studying cell-specific HIV expression. In this transgenic rat line, intracranial injections of the pro-inflammatory cytokine Tumor Necrosis Factor alpha (TNFα) into iHIV animals elicits an increase in Nanoluciferase activity. Here we present characterization data of the CX3CR1-CreERT2 and iHIV rats. Collectively, our data identify a potential model for studying HIV neuropathogenesis in the rat brain. This work was supported by the Intramural Research Program at NIDA/NIH.

577. Light-Induced Nrf2 Knockout Mice as Atrophic Age-Related Macular Degeneration Model & the Treatment with Nanoceria Laden Injectable Hydrogel

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The elevated oxidative stress and associated reactive oxygen species (ROS) accumulation are hallmark in the induction and progression of age-related macular degeneration (AMD). We introduced a new murine model using Nrf2 knock out mice with light-induced damage to the retina to simulate the symptom of atrophic AMD. This animal model exhibited phenotype of photoreceptor degeneration, impairment of retinal function, accumulation of ROS, and inflammation reaction in relatively shorter time. The nanoceria acted as a robust antioxidant and water soluble with polysaccharide coating. The delivery of nanoceria from alginate-gelatin based injectable hydrogel enabled homogeneous distribution of nanoceria and prevented the aggregation of nanoceria

on retina pigment epithelium cells. Nanoceria laden injectable hydrogel protected retina from further oxidative damage and inflammation. The technique has potential to translate to clinic for the treatment of AMD.

578. Development of an Effective Technology for Inductive Differentiation from Bone Marrow-Derived Mononuclear Cells to Neuroprotective Microglia

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[Background]Microglia are universally known as immunocompetent cells in the central nervous system and have been recognized as an important target for treatment of various neurological disorders. Microglia are classified into two major phenotypes, comprising cytotoxic and neuroprotective types. Therefore, the selective induction of neuroprotective microglia enables us to apply them to treat neurological diseases. [Objective]To establish a technology to induce neuroprotective microglia from bone marrow-derived mononuclear cells (BM-MNCs) effectively for treatment of neurological disorders. [Material and methods](1) Mixed glial cells were isolated from postnatal C57BL/6 mice, and were cultured for two weeks. Subsequently, primary microglia were isolated from mixed glial cell cultures and were incubated with GM-CSF or IL-4 for further three days. (2) BM-MNCs were isolated from adult C57BL/6 mice and were cultured with GM-CSF only, both GM-CSF and IL-4, or GM-CSF adding on IL-4 at day three, for seven days. In those cultured microglia and BM-MNCs, the usability for cell therapy were evaluated at the points of proliferation rate, morphological changes and gene expression profiles. [Results](1) GM-CSF-activated microglia showed significant proliferation. Gene expression of CD86, an inflammation marker, was upregulated in GM-CSF group. In contrast, gene expression of CD206, a neuroprotective marker, was upregulated by IL-4 stimulation. (2) BM-MNCs showed an increased proliferation by GM-CSF and an effective differentiation to neuroprotective microglia by adding on IL-4 to GM-CSF. [Conclusion]We here demonstrate the therapeutic potential of BM-MNCs using a novel differentiation technique generating neuroprotective microglia, which could be expected for treatment of neurological diseases in future.

579. Development and Characterization of a Novel Choroidal Endothelial Cell Line for Studying Age-Related Macular Degeneration (Amd)

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Age-related macular degeneration (AMD) is a common, blinding disorder affecting the central visual field. Prevalence of AMD is as high as 18% in elderly individuals. Whereas end-stage AMD involves degeneration of light sensing photoreceptor cells and the retinal pigment epithelium (RPE), recent clinical, histopathological, and molecular studies have revealed that degeneration of the choriocapillaris—the vascular supply for photoreceptor cells and RPE—is an early indicator of disease. These findings suggest that protecting or replacing choriocapillaris endothelial cells early in the disease process may prevent progression to end stage, blinding disease. However, to date there is a lack of tools for studying choroidal repair in AMD. In this study, we obtained eyes from a one month old female donor. Cultures of choroidal cells were prepared by proteolytic digestion and isolation of CD31-positive cells using antibody-conjugated magnetic beads. Cells were grown in Endothelial Cell media and were immortalized by transfection with lentiviral vectors containing the human CDH5 promoter driving expression of telomerase and the SV40 T-antigen. Both vectors also contained the blasticidin resistance gene. Blasticidin-resistant cells were subcultured and expanded. Cells derived from this protocol express CD31, CD34, and von Willebrand factor, and form tubes in matrigel. This immortalized human choroidal endothelial cell line will be useful in reproducibly evaluating conditions for reconstructing the choroid in humans with AMD.

580. Assessment of Intracerebroventricular AAV9-hTPP1co Efficacy in Batten Mice Using a Digital Vivarium (Vium, Inc.)

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CLN2 disease, a form of Batten disease, is a neurodegenerative lysosomal storage disorder caused by mutations that decrease activity of the soluble enzyme, tripeptidyl-peptidase-1 (TPP1). The disease is characterized by early onset at 2-4 years of age with seizures and ataxia before death by mid-childhood. Enzyme replacement therapy (ERT) with recombinant TPP1 (Brineura®, BioMarin Pharmaceuticals) was recently approved and is administered as a biweekly infusion into the lateral ventricles via a permanently implanted device. Repeat infusions are necessary due to the short half-lives of recombinant enzymes. Thus, there remains a significant unmet need for therapies that can provide sustained TPP1 activity in patients without the high burden associated with repeat administration. To address this, we conducted a study to

assess the efficacy of intracerebroventricular (ICV) AAV9 encoding human *TPP1* in a mouse model of CLN2 disease, the TPP1^{m1J} mutant. The TPP1^{m1J} mice recapitulate most features of the human disease, such as shortened lifespan, seizures, or abnormal gait. Objective monitoring of the neurobehavioral function in this model is, however, challenging as they are prone to stress-induced fatal seizures after 3 months of age and do not present any measurable deficit before this age. To circumvent this, we used an unbiased non-invasive full-time monitoring system in a digital vivarium (Vium, Inc.). This technology allows continuous recording of activity, breathing rate, and circadian rhythms in the home cage of single-housed mice without any operator manipulation. Seven-week-old mice received a single dose of 3×10^{11} GC of AAV9-hTPP1co in the right cerebral ventricle; vehicle-injected WT littermates and TPP1^{m1J} mice were used as controls (10 to 13 animals per group). Recordings started 2 weeks after treatment and continued until sacrifice or unscheduled death. At study termination, 16 weeks post-ICV injection, necropsies were performed and brain and liver were sampled. All untreated KO animals had tremors, abnormal gait, or seizures, whereas 54% of vector treated KO animals were unremarkable throughout the study. Treatment produced supraphysiologic TPP1 activity in liver, and increased the lifespan of KO mice. At the terminal endpoint (age 23 weeks), 62 % of treated KO versus 0% of untreated KO mice were alive. Untreated KO mice showed decreased activity during the dark phase while vector-treated animals presented circadian motion profiles that were identical to WT until the end of the study. Taking advantage of the repeated measurements allowed by the digital vivarium, the statistical analysis of nightly motion speed using linear mixed effect modeling showed a significant therapeutic benefit in vector-treated KO mice starting at 14.7 weeks of age, while the average age at first clinical event was 16.1 weeks. This demonstrates that KO mice have neurobehavioral impairment that precedes the onset of grossly visible clinical signs and that treatment prevented this impairment. Corresponding to the rescue of neurobehavioral parameters, astrocytosis, a marker of neuroinflammation, was decreased in brain tissue of vector-treated animals. Altogether, these results demonstrate the therapeutic efficacy of AAV9-mediated gene therapy to prevent neurobehavioral manifestations, increase survival, and rescue the neuroinflammation related to CLN2 disease. Using a state-of-the art digital vivarium, we also demonstrated the presence of circadian rhythm abnormality in presymptomatic mice providing objective early efficacy readout in this model. This pilot study suggests that a single administration of AAV9 in the cerebrospinal fluid could be an alternative to repeat ERT for the treatment of CLN2 disease.

581. Non-Invasively Silencing and Monitoring Machado-Joseph Disease

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Machado-Joseph disease (MJD) is a genetic neurological disorder associated with the expansion of a (CAG)n trinucleotide in the MJD1/ATXN3 gene, which translates into an expanded polyglutamine (polyQ) tract within ataxin-3, conferring toxic properties to this protein and resulting in severe clinical features. We and others demonstrated that silencing mutant ataxin-3 mRNA (mutATAX3) using RNA interference upon intracranial injection of viral vectors targeting mutATAX3 decreases the neuropathological abnormalities in rodent models. However, this is an invasive procedure, associated with several adverse effects. Therefore in this study we aimed at developing an adeno-associated virus (AAV)-based system that enables: non-invasive delivery of RNA interference-based treatments to the brain, specific silencing of mutATAX3 and alleviation of MJD by intravenous (iv) injection. We made use of AAV serotype 9 (AAV9), a non-pathogenic viral vector that has the ability to bypass the BBB and transduce the CNS of mammals. AAV9 vectors encoding an artificial microRNA that targets the mutATAX3 mRNA (AAV9-mirATAX3) were firstly generated. Their efficacy and specificity were tested in neuronal cells and the therapeutic potential was then evaluated in a severely-impaired transgenic mouse model. Mice were intravenously injected at postnatal day one (PN1); were submitted to behavioral tests at three different ages (PN35, 55, and 85) and sacrificed at PN95. At PN75, animals underwent in vivo Magnetic resonance imaging and spectroscopy (MRI/MRS) to evaluate morphological and metabolic changes of cerebellum. AAV9-mirATAX3 vectors efficiently spread throughout the brain, transducing regions affected in MJD. AAV9-mirATAX3's treatment reduced the number of protein aggregates and cerebellar neuropathology, leading to significant improvements in all behavioral tests. Moreover, MRI/MRS data indicated that mirATXN3 treatment ameliorates the levels of some important metabolites in the cerebellum, e.g. N-acetylaspartate (NAA), Inositol, and Choline. Overall, this study provides compelling evidence that a single iv injection of AAV9-mirATAX3 is able to transpose the BBB, silence mutant ataxin-3 and alleviate MJD. To our knowledge, this is the first time that a non-invasive and allele-specific silencing approach produces a significant positive impact in a neurodegenerative disorder.

References:

Alves S, et al. PLoS ONE 2008 Oct 8;3(10):e3341.
Alves S, et al. HMG 2010 15;19(12):2380-94.
Nobrega et al. (2013) PLoS ONE 8(1): e52396.

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879. AAV Targeting of the Peripheral Nervous System

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Recombinant adeno-associated virus (AAV) have been extensively used for targeting gene therapies to the central nervous system (CNS) and have repeatedly demonstrated safety and efficacy in animal models and in humans to transduce a wide range of cell types. The peripheral nervous system (PNS) is a vast collection of neurons and glial cells that are not inhibited by the blood-brain-barrier like the CNS. As such, AAV serotype targeting of the PNS may be different than that of the CNS. The PNS can broadly be divided into the somatosensory and autonomic nervous systems, that latter of which can be further divided into the sympathetic, parasympathetic and enteric nervous systems. Studies have shown that AAV can transduce PNS neurons by directly injection naturally occurring AAV serotypes and variants into specific PNS tissues, however, in the interest of identifying AAV variants that widely target the PNS with a single dose, direct injection is not a feasible route of delivery. Here we present a comprehensive study that identifies AAV capsids that target the peripheral nervous system following systemic delivery. Adult C57BL/6 mice were injected via tail-IV with naturally occurring or engineered capsids carrying a self-complementary CBh-GFP reporter gene. All virus were produced by the UNC Vector Core and formulated in PBS with 5% sorbitol. Naturally occurring capsids AAV1, 2, 4, 5, 6, 8 and 9 were analyzed. Engineered capsids included AAV8G9, PHP.B and a collection of novel capsids that were developed by our lab using AAV capsid DNA shuffling and directed evolution for CNS gene transfer. Mice were harvested 4-weeks post-injection and the distribution and neurotropism of gene expression was determined by immunofluorescence staining of GFP expression within various peripheral nervous system networks. Tissues analyzed include trigeminal and dorsal root ganglion (sensory nervous system), sympathetic chain ganglia and coeliac and mesenteric ganglion (sympathetic nervous system), submandibular ganglion (parasympathetic nervous system), and myenteric and submucosal plexus in the intestine (enteric nervous system). Results from the naturally occurring serotypes showed that systemic delivery of AAV8 and AAV9 resulted in widespread transduction of neurons and glia in all peripheral nervous tissue examined. Systemic delivery of AAV1, 5 and 6 failed to transduce cells within peripheral ganglia and delivery of AAV2 resulted in the rare transduction of peripheral neurons. Interestingly, while AAV4 failed to transduce neurons in all peripheral nervous tissue examined, there was a consistent transduction of a non-neuronal cell population and studies are ongoing to identify these cells. Both AAV8G9, which is an AAV8 and 9 chimeric capsid, and PHP.B, a capsid variant of AAV9, widely transduced PNS neurons and depending on tissue type with varying efficiency as compared to the parent capsids. Several clones from our novel library showed a range of neuronal tropism within the PNS, although not the extent of AAV8, AAV9, AAV8G9 and PhP.B. Analyses of additional engineered capsids are ongoing and will be presented. Engineered capsids that also target the PNS might be useful alternatives for patients seropositive for AAV8 or AAV9. Overall, these results provide a basis for the development of novel therapies for diseases with global PNS dysfunction and autonomic dysfunction, such as hereditary sensory and autonomic neuropathies.

Oligonucleotide Therapeutics II

582. Abstract Withdrawn

583. Exploration of Gene Optimization and scFv-Fc Reformatting as Strategies to Increase *In Vivo* Expression Levels of DNA-Encoded Monoclonal Antibodies (DMAbs) against Zika Virus

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Inovio's DMAb ™ platform offers a novel approach to generate mAbs *in vivo* by transfecting mAb-encoding plasmid into skeletal muscle cells using electroporation (EP). DMAbs can achieve high serum levels and show protection comparable to purified mAbs in flu and pseudomonas murine challenge models (Patel *et al.* 2017, Elliott *et al.* 2017). Working toward clinical application, we have focused our efforts on further increasing the *in vivo* expression levels of DMAbs through formulation, administration, and compositional changes at the nucleotide and amino acid level. Here we present two antibody modification strategies for DMAbs targeting the Zika virus: gene optimization and scFv-Fc reformatting. The gene optimization method consisted of selecting two full length Zika DMAb sequences and optimizing via six different algorithms. Multiple parameters affecting transcription and translation, such as codon usage, GC content, cryptic splice sites and mRNA secondary structure are weighted in proprietary multivariate regression algorithms. Much of the data referenced, however, was generated using in vitro expression systems. We sought to find an algorithm most suited to the in vivo expression of our Zika DMAbs. BALB/c mice (n=5) were administered with 100 μg of plasmid DNA in one treatment site through intramuscular delivery followed by electroporation. Serum levels and normalized binding of DMAbs were quantified by ELISA at day 7. For ZKDMAB-1, Algorithm 1 gave the highest expression at 18 ug/ml. For ZKDMAB-2, Algorithm 2 gave the highest expression of 3.5 ug/ml. Consistently, both DMAbs optimized by Algorithm 6 exhibited the lowest or no expression in vivo. In most cases, binding by ELISA was retained, however several algorithms saw a decrease for ZKDMAB-1, suggesting that protein folding or conformation of the expressed DMAb could have been affected by the nucleotide sequence. Further investigation is warranted. Additionally, we tested scFv-Fc conversion, which intends to promote heavy chain - light chain pairing and tissue penetration. Two Zika DMAbs were chosen, and from them multiple constructs were generated. They differed in

their choice of linker molecule and the orientation of the VH-VL. Converting DMAbs from a full length antibody to scFv-Fc resulted in an increase in murine expression of up to 6 fold compared to the original DMAb. For ZKDMAB-1, expression of the four formats tested ranged from 16 ug/ml down to 8 ug/ml and favored the (G4S)3 linker in the VH-VL orientation. ZKDMAB-2 saw highest expression reach 12 ug/ml using the (G4S)3 linker in the VL-VH orientation. Importantly, modifications made to the majority of DMAbs retained antigen binding. Through these changes we are able to increase the *in vivo* expression levels, but not sacrifice the biology of the original mAb clone. These data demonstrate the obvious benefit of gene and protein modulation when designing DMAbs for gene therapy applications.

584. Nuclease-Activated Probe Technology for Cancer Detection

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Nucleases have been proposed as powerful biomarkers for cancer detection. Advantages of nucleases for cancer detection include: ***1)*** their elevated expression in most cancer cells (including those cancer cells in blood implicated in metastasis that have undergone an epithelial-to-mesenchymal transition - EMT), and ***2)*** their enzymatic activity, which can be exploited for signal-amplification in detection methods. We recently developed a novel technology to enable the rapid and robust detection of cancer-derived nucleases in patient blood (**Fig. 1**). This technology, Nuclease Activated Probe (NucAP), is based on chemically-modified, quenched fluorescent probes that are activated by cancer nucleases but not nucleases present in human serum (e.g. RNase A). Proof-of-concept studies to date exploring this approach have focused on circulating tumor cell (CTC)-derived intracellular nucleases from advanced (stage IV) breast cancer patients.[1] These studies have identified 3 probes (dsDNA, ssDNA and 2′F-RNA) which exhibited robust performance in distinguishing patients with stage IV breast cancer from healthy controls. Given the difficulty in capturing CTCs from blood, more recently, we have begun to evaluate the NucAP technology with cancer-derived nucleases secreted in serum/plasma. To date, we have observed elevated cancer-derived nuclease activity in both breast and pancreatic cancer cell lines grown in culture and patient serum. Elevated nuclease activity in patient serum was consistent with the presence of DNAse-like nucleases. In contrast, DNAse-like nuclease activity was reduced in the plasma samples of prostate cancer patients compared to healthy donors and inversely correlated with disease stage. Proteomic analysis of the prostate cancer plasma samples has revealed the identity of several DNAse-like nucleases whose levels decrease with disease progression. Current studies are focused on confirming the identity of the specific nucleases and determining the mechanism for reduced nuclease activity in prostate cancer. We are also investigating potential serum vs. plasma collection effects on assay turnout. In summary, the NucAP approach is simple (which can facilitate point-of-care), inexpensive, and can be applied to both CTC-derived intracellular nucleases as well as cancer-derived nucleases secreted in serum without the CTC capture step. The implications of these findings could be transformative for several reasons. ***1)*** they could lead to the development of effective assays for the early detection of various cancers including breast, pancreatic and prostate cancer and for monitoring treatment response that is both straightforward and cost effective. ***2)*** they provide proof-of-principle for a new point-of-care platform that would enable clinicians to capitalize on the vast potential of nucleases as biomarkers for cancer diagnostics. 1. Kruspe, S. et al. Rapid and Sensitive Detection of Breast Cancer Cells in Patient Blood with Nuclease-Activated Probe Technology. *Mol Ther Nucleic Acids* **8**, 542-557 (2017).

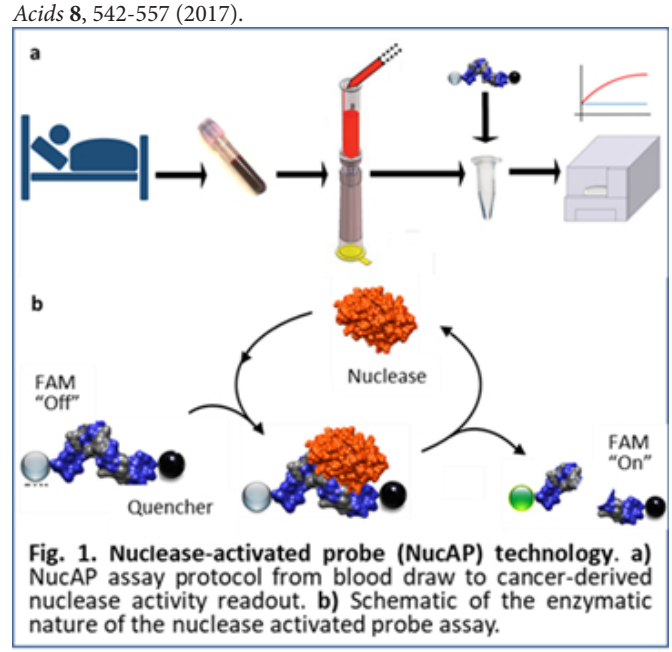

Fig. 1. Nuclease-activated probe (NucAP) technology. a) NucAP assay protocol from blood draw to cancer-derived nuclease activity readout. **b)** Schematic of the enzymatic nature of the nuclease activated probe assay.

585. Enhanced Levels of FKRP Following Oligonucleotide Targeting as a Therapy for LGMD2I

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Limb-girdle muscular dystrophy 2I (LGMD2I) is caused by mutations in the gene for fukutin-related protein (FKRP) resulting in the loss or reduction of functionally glycosylated alpha-dystroglycan (F-a-DG). This loss of F-a-DG is the primary characteristic of a subset of muscular dystrophies known as dystroglycanopathies. Although patients and mutant mouse models with FKRP mutations exhibit muscle fibers lacking detectable F-a-DG, a small number of fibers expressing strong F-a-DG, described as revertant fibers, are present. The presence of these fibers suggests at least a partial function of mutant FKRP. Taken together with recent results in our lab demonstrating an increase in F-a-DG using AAV delivery of mutant FKRP, it appears possible to restore levels of F-a-DG through overexpression of endogenous mutant FKRP. Here we explore the use of antisense oligonucleotides (AON) for enhanced mRNA stability of FKRP to increase endogenous levels. Preliminary results of optimized sequences demonstrate some significantly increased levels following intramuscular injections.

Additionally, in vitro targeting of similar regions in the 3'UTR in human FKRP show some significantly increased levels of expression. Short-term and long-term treatments of vivo-PMOs targeting the 3'UTR of FKRP mRNA demonstrate increased levels in skeletal muscle following three weekly treatments as well as treatment every two weeks for 3 months. Overall, our results indicate that upregulation of FKRP through AON targeting can be achieved in vitro and in vivo and provides an alternative approach to gene overexpression in treating dystroglycanopathies with mutations remaining at least partial function.

586. Synergistic Effects of Small Therapeutic Rnas Targeting Transcriptional Factors in Glioblastoma (GBM)

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Glioblastoma multiforme (GBM) is the most common aggressive malignancy of the central nervous system (CNS) with less than 5% of survival rate. Despite of the great efforts to find effective therapeutic options, still this type of tumor remains incurable. To improve the survival rate and the therapeutic index, the selection of the well-tailored therapeutic approach is critical such as targeted therapeutics. For targeted delivery of therapeutic modalities, cancer specific receptors are very attractive targets. The cell surface receptor tyrosine kinase receptor of platelet derived growth factor receptor α (PDGFRα) is selectively expressed on embryogenic development stage, but the expression of PDGFRα is very limited in smooth muscle and intestinal mucosa cells after postnatal. In this aspect, PDGFRα is very attractive target for targeted therapeutics. In the progression of malignancy of GBM, the overexpression of PDGFRα plays an important role in tumor cell growth, proliferation, and migration. Aiming for targeted therapeutics in GBM for the delivery vehicles, RNA aptamers were selected against PDGFRα throughout the SELEX method. The isolated RNA aptamers, termed PDR3, showed the high binding affinity of 24.8nM by Biacore T100. The PDR3 also showed the internalization in human glioblastoma cells, U251-MG. For therapeutic payload, the siRNA against the transcriptional factors signal transducers and activator of transcription 3 (STAT3) was selected based on the regulatory axis of PDGFRα and STAT3. For targeted delivery of therapeutic modalities, the nucleic acid chimers, PDR3-siSTAT3 (small inhibiting STAT3) were constructed. The target gene expression was analyzed in treatment groups of PDR3-siSTAT3 by qPCR. The gene expression of STAT3 showed that 55% reduction by PDR3 aptamer themselves and the chimera of PDR3-siSTAT3 showed the synergistic suppression of STAT3 in 48 hours. Furthermore, the inhibition of STAT3 led to the regulation of Jmjd3 (KDM6B) and repressed the neural differentiation genes. Based on these results, targeting transcriptional factor STAT3-PDGFRα axis is suggested as synergistic anti-tumor therapeutics in GBM.

587. Translation Enhancing Effects of Sequence Specific Oligonucleotides Targeting 5'UTRs of Selected Tumor Suppressors

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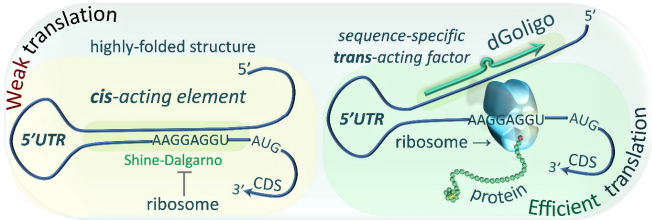
Background: Translation initiation is a key rate-limiting step in a cellular protein synthesis. The frequently reported lack of correlation between various mRNA and protein levels in cancers suggests that translational control can be a promising target for new therapeutics regulating mechanisms of protein synthesis. Cellular microRNAs and synthetic siRNAs are the most recognized regulatory molecules acting via RNAi. Surprisingly, recent studies have shown that the interfering RNAs may also activate gene transcription through the newly discovered phenomenon of small RNA-induced gene activation (RNAa). Thus far, the small activating RNAs (saRNAs) inducing RNAa have only been demonstrated as promoter-specific transcriptional activators. **Findings**: We demonstrate that oligonucleotide-based *trans*-acting factors can also specifically enhance gene expression at the level of initiation of protein translation by acting at sequence-specific targets within the messenger RNA 5'-untranslated region (5'UTR). We show that oligonucleotide-based trans-acting factors termed dG*oligos (dGs)*, can also specifically enhance gene expression at the level of protein translation by acting at sequence-specific targets within mRNA 5'-untranslated regions (5'UTRs) of *THRB* suppressor. The *in vitro* translation efficiency of reporter constructs containing alternative TRβ1 5'UTRs was increased by up to 55.8-fold following exposure to specific dG*s*. Complementary *in vivo* study showed that dGs can enhance TRβ1-5'UTR-mediated translation up to 4.8-fold. This method was successfully applied to enhance translation of another *CDKN2A* suppressor. We show that the most folded 5'UTR has higher translational regulatory potential when compared to the weakly folded TRβ1 variant suggesting that the strategy may be especially applied to enhance protein synthesis from translationally non-active or less-active transcripts containing long strongly folded 5'UTRs. dG*s* can serve as molecular switches to translationally active conformation of folded 5'UTRs leading to efficient translation of target mRNAs. **Significance**: This study represents the first strategy for gene-specific translation enhancement using selective *trans*-acting factors designed to target specific 5'UTR *cis*-acting elements. This developmental strategy may complement other available methods for gene expression regulation including gene silencing and may find its use in enhancement of genes frequently silenced in cancers to support molecular targeted therapies.



588. Systemic Expression of Translatable Circular Rnas Using Recombinant AAV Vectors

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Circular RNAs (circRNAs) are stable, covalently closed RNAs that are abundantly expressed and evolutionarily conserved across species. Several possible functions ranging from miRNA and RNA binding protein sponges to regulators of transcription and translation have been proposed. CircRNAs are more long-lived than their linear counterparts, due to the lack of free ends and consequently resistance to exonucleases. Accordingly, circRNAs may accumulate over time in non-dividing tissues. Here we describe the design and characterization of recombinant adeno-associated viral (AAV) vectors packaging transgene cassettes containing intron sequences that backsplice to generate circularized RNA transcripts. Using a split GFP transgene, we demonstrate that the efficiency of circRNA formation varies with different introns and in a cell type-specific manner in vitro. Further, translation from circRNA is efficiently driven by an internal ribosomal entry site (IRES). Upon injecting AAV vectors encoding circRNA in mice through the systemic route, we observed robust transgene expression in the heart, but low transduction in the liver. In addition, expression in the murine brain was restricted to astrocytes following intracranial administration, while intravitreal injection in the eye yielded robust transgene expression across multiple retinal cell layers. These results highlight the development of a new AAV-based toolkit to study circRNA formation in mouse models and tissue-specific regulation of RNA circularization, as well as exploring the potential for developing therapeutic platforms using this approach.

589. Abstract Withdrawn

590. Combination Therapy Using Erythropoietin and Tmprss6-Antisense Oligonucleotides Improves Anemia, Iron Overload and Splenomegaly in Beta-Thalassemia

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Beta-thalassemia intermedia (BTI) is characterized by ineffective erythropoiesis, anemia, splenomegaly and systemic iron overload. In theory, erythroid stimulating agents (ESA), such as erythropoietin (EPO), could improve anemia by increasing the production of red blood cells (RBC), but with the negative side effects of exacerbating the splenomegaly and increasing iron absorption. However, we postulated that, in presence of Epo, iron restriction will still improve the anemia, but this time with beneficial consequences of reducing the splenomegaly and iron overload. To increase Epo levels in a mouse model of BTI ($Hbb^{th3/+}$), we used an ex vivo technology where primary dermal fibroblasts, transduced with an adenovirus, expressed high levels of Epo (TARGTEpo) once implanted in $Hbb^{th3/+}$ and wild-type (WT) control mice. Pharmacokinetic studies indicated that between 10e5, and 1x10e6 implanted cells were sufficient to significantly increase Epo synthesis (up to 47.6% and 90% more, respectively, in $Hbb^{th3/+}$ and WT animals). In WT mice, one week after implantation, hemoglobin (Hb) levels increased, on average, 3.3g/dL from the baseline level. In $Hbb^{th3/+}$ mice, Hb levels rose up to 11.7g/dL and remained elevated until the end of the 6-week treatment (compared to 7.7g/dL in controls). As expected, stimulation of erythropoiesis led to worsening of splenomegaly and suppression of hepcidin, the hormone that controls dietary iron absorption. This prevented the beneficial effect of erythroid-mediated consumption of iron stored in excess in organs of $Hbb^{th3/+}$ animals. TARGTEpo was then combined with low iron diet or antisense oligonucleotides targeting the *Tmprss6* mRNA (*Tmprss6*-ASO; the last treatment only for $Hbb^{th3/+}$ animals). It has been shown that inhibition of *Tmprss6* increased expression of hepcidin, which decreased iron absorption and limited erythroid iron intake. This improved ineffective erythropoiesis, splenomegaly and iron overload in $Hbb^{th3/+}$ mice. In WT animals, after three weeks, the combination of TARGTEpo with low iron diet significantly reduced Hb levels (-40%), RBC number (-38%) and reticulocytes (-80%), when compared to animals overexpressing Epo and receiving normal iron diet, indicating that the stored iron was insufficient to support the increased erythropoiesis. In contrast, $Hbb^{th3/+}$ animals on iron deficient diet or treated with *Tmprss6*-ASO in presence TARGTEpo showed improvement of anemia up to the end of the treatment (6-weeks). Strikingly, in these animals the improvement in the anemia was associated with reduced splenomegaly. Furthermore, preliminary data indicate that organ iron concentration improved or normalized compared to control $Hbb^{th3/+}$ mice. In particular, in animals treated with *Tmprss6*-ASO and TARGTEpo the Hb levels increased significantly (on average 4.3 g/dL, reaching levels of 12g/dl), and corresponding to 36% more that the baseline levels in $Hbb^{th3/+}$ control animals (7.7g/dL) and +25% more compared to $Hbb^{th3/+}$ mice treated with *Tmprss6*-ASO alone (9.0g/dL). The splenomegaly was reduced compared to baseline levels (-35%) and to animals treated only with TARGT-Epo (-52.4%). Analysis of pathways that control iron absorption and erythropoiesis are in progress. In conclusion, we propose that, in beta-thalassemia, careful administration of ESA and iron restrictive agents could improve the anemia and the splenomegaly, and quickly resolve the iron overload.

591. Long Non-Coding Rna Transcriptional Modulation of Vegf-A during Hypoxia

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The role and function of long non-coding RNAs (lncRNAs) in modulating gene expression is becoming apparent. Vascular endothelial growth factor A (VEGF-A) is a key regulator of blood vessel formation and maintenance making it a promising therapeutic

target for activation in ischemic diseases. Small ncRNAs directed to the VEGF-A promoter have previously been observed to specifically activate VEGF-A expression. Little is known regarding the mechanism of action, whereby small and/or long ncRNAs modulate VEGF-A transcription. In this study, the role of two antisense VEGF-A lncRNAs, RP1-261G23.7 and EST AV731492, were interrogated in both normoxia and hypoxia treated endothelial cells. We find here that both lncRNAs are concordantly upregulated with VEGF-A in hypoxia and appear to localize to the nucleus. LncRNA over-expression has no effect on VEGF-A expression whereas knocking-down of the lncRNA transcripts resulted in the downregulation of VEGF-A expression. Both lncRNAs were shown to be polyadenylated and to localize to the VEGF-A promoter and upstream elements in a hypoxia dependent manner. Collectively, these data suggest that VEGF-A antisense lncRNAs, RP1-261G23.7 and EST AV731492, may function as VEGF-A promoter enhancer elements, possibly by acting as a local scaffolding for proteins and small RNAs to tether. Notably, targeting of these transcripts, to enhance VEGF-A expression during hypoxic conditions, may prove beneficial when developing novel treatment strategies for diseases resulting from oxygen deficiency.

592. Manipulating the Vector Interactome to Enhance Gene Delivery

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Delivery of nucleic acids for gene therapy suffers from a lack of efficacy due to poor uptake and rapid degradation of nucleic acids by the body. Non-viral delivery vehicles offer significantly improved efficacy over delivery of DNA/RNA alone, but still suffer from poor cellular uptake and/or necessary compartment localization (e.g. the nucleus for DNA or the ribosome for RNA). Understanding the barriers that reduce the efficacy of the delivery vehicle can guide us on ways to improve efficacy through manipulation of the target cell rather than the vector. The lung presents a particularly challenging target with complex extracellular barriers such as extensive vascularization, blood, and mucus. However, even in airway cell line cultures that lack mucus/blood, nonviral vectors are inefficient which suggests that intracellular barriers are important. Currently, nucleic acid nanoparticles (NNPs) meet the criteria of a vector that can deliver RNA or DNA to non-dividing airway epithelial cystic fibrosis (CF) cells with no measurable toxicity in animals and CF patients. Previously, NNP interaction with nucleolin at the cell surface was found to be a major step in cellular uptake and trafficking. In studies of the mechanisms of NNP uptake, it was found that activating the glucocorticoid receptor with cortisone during NNP transfection increased the internalization of nucleolin and NNP uptake by 3-4 fold. To identify more potential protein targets, we used immunoprecipitation and mass spectrometry to identify the particle cellular interactome. Examination of primary CF cells from 7 different patient donors revealed important interactors involved in particle uptake such as non-erythrocytic spectrin alpha 1 (SPTAN1), heat shock protein 90 (HSP90), and adenomatous polyposis coli (APC). We examined the effect of knockdown or pharmacological manipulation of the NNP interactome on delivery of a NNP previously used in CF patients, DNA complexed with peptides of 1 L-cysteine and 30 L-lysines conjugated by maleimide linkage to 10kDa PEG (10kPEG-CK30). Our DNA nanoparticles (DNPs) contained a DNA plasmid to express firefly luciferase driven by an ubiquitin B promotor, and we conducted our studies in well differentiated airway epithelia cultured at air-liquid interface. We found that gene transfer can be markedly enhanced by knockdown of APC or SPTAN1 by 82.4 + 6.2% vs. scrambled control (with no cell death detected, 12 replicates of cells from 7 different donors across 5 separate experiments). Two days following treatment with scrambled control, DNPs achieved $2.26 \times 10^6 + 1.50 \times 10^6$ ILU/mg protein, while knockdown of SPTAN1 shRNA or APC1 shRNA resulted in $11.2 \times 10^7 + 3.32 \times 10^6$ and $81.9 \times 10^7 + 3.89 \times 10^6$ ILU/mg protein, respectively. Pharmacological manipulation during transfection resulted in increases of ~10-100 fold versus vehicle controls. For example, co-treatment with 300nM cortisone resulted in $9.87 \times 10^6 + 3.8 \times 10^6$ ILU/mg protein versus $1.26 \times 10^6 + 0.56 \times 10^6$ ILU/mg protein from vehicle treated control. These results support our hypothesis that manipulation of intracellular interactions with NNPs significantly enhances gene delivery. Pharmacological co- treatment during application of the vectors in humans (i.e. cortisone treatment during DNP administration) is a feasible approach to enhance gene transfer efficacy in the clinic.

593. New Generation Cd44-X-Aptamers for Targeting Human Ovarian Cancer Cells

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CD44 is a multi-structural and multi-functional cell surface molecule involved in cell-cell interactions, cell adhesion and migration, and is a receptor for hyaluronic acid. CD44 participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis. Development of CD44 aptamers specifically targeting CD44 can be used for targeting CD44 positive cells, including cancer stem cells and for drug delivery. On the basis of the primary sequences of several of our previously selected thioaptamers (TAs) and observed variations, we developed a bead-based X-aptamer (XA) library by conjugating drug-like ligands (X) to the 5'-positions of certain uridines on a complete monothioate backbone. We selected an XA with high affinity to CD44 hyaluronic acid binding domain (HABD) from a large combinatorial X-aptamer library modified with a drug (N-acetyl-2,3-dehydro-2-deoxyneuraminic acid, ADDA) and demonstrated an enhanced binding affinity for the CD44 protein by up to 23-fold. The selected CD44 X-aptamers (both amine form and ADDA form) also show enhanced binding affinity to CD44 over-expressing human ovarian cancer IGROV cells. Based on secondary structure predictions of CD44 performed using MFold, we identified several binding motifs and smaller constructs of various stem-loop regions. Among our several identified binding motifs, X-aptamer motif 3 and motif 5 showed enhanced binding affinity to CD44 over-expressing human ovarian cancer IGROV cells with ADDA form, compared to the binding affinities of amine form and scrambled sequence (Figure 1). X-aptamer motif 3 and motif 5 had shown the

same pattern of cell binding as that of anti-human CD44 antibody. The effect of ADDA as binding affinity enhancer is location dependent within the aptamer. The process to find optimal position of the ligand was part of our X-aptamer selection. The incorporation of ADDA not only expands the XA's chemical diversity but also the surface area of binding, thus the XA can also offer enhanced specificity.

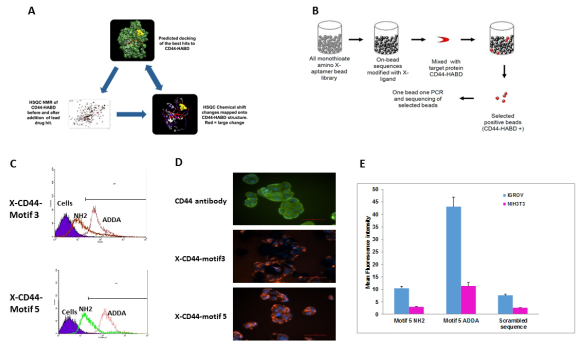

Figure 1. Selection and validation of CD44 X-aptamer. Drug Hit Validation (A). Bead-based CD44 X-aptamer Selection (B). Validation of binding affinity of selected motifs by flow cytometry (C) and Immunofluorescence (D). Specific binding of X-CD44-motif 5 to CD44 expressing IGROV cells (E).

594. Exploiting Gene Amplification in Cancer Using Triplex Formation as a Novel Therapeutic Strategy

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Gene amplification is a major mechanism driving the malignant transformation and progression of human cancers by mediating changes in the expression of genes that are involved in normal cell growth and survival pathways. Its significant impact combined with the identification of numerous amplified cancer-driver genes via recent advances in DNA sequencing technology, has positioned gene amplification as a priority candidate for anticancer drug development. Several targeted cancer therapies have been designed to block the oncogenic activity of amplified genes. However, the majority are aimed at the overexpressed protein products and their clinical efficacy is often hampered by drug resistance. As an alternative, we developed a drug platform that directly targets the amplified chromosomal DNA and exploits the cell's own DNA damage response machinery to induce tumor specific apoptosis. Our approach has been to develop triplex-forming oligonucleotides (TFOs) that bind sequence specifically to unique polypurine sites of the amplified gene. The TFOs bind in the major groove of duplex DNA causing a distortion of the alpha-helix, which can perturb DNA replication fork progression, eventually resulting in fork collapse and induction of DSBs. We have uncovered the novel finding that TFOs can only induce apoptosis when multiple triplex structures are formed, since the nucleotide excision repair (NER) pathway is capable of efficiently repairing a low level of damage induced by the presence of one or two structures. *HER2* gene amplification found in ~25% of breast cancers provides an opportunity to use TFOs to create multiple apoptosis inducing triplexes in the cancer cells and not health cells, which lack HER2 amplification. We have evaluated the efficacy of HER2-targeted TFOs using cell growth and apoptosis assays and have determined that these molecules are particularly effective in eliciting tumor specific apoptosis in HER2-amplified breast and ovarian cancer cell lines. Furthermore, these molecules have the ability to suppress the growth and metastasis of human HER2-positive cancers in xenograft mouse models with a drug efficacy comparable to the currently used targeted therapy, trastuzumab. Moreover, we demonstrate that the NER protein, XPD mediates the survival-death decision in response to triplex-induced DNA damage via a p53-independent mechanism. This study introduces a new paradigm for gene-targeted cancer therapy, which can have enormous impact on the field of precision medicine.

595. Cancer-Specific Promoters for Mitochondrially-Targeted p53-BH3 Gene Therapy in Ovarian Carcinoma

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Though considerable efforts have been poured into cancer therapy research, huge hurdles remain in order to conquer the disease—particularly genetic heterogeneity among tumor cells and the development of drug resistance. Tumor suppressor p53, once touted as a highly promising gene therapy, has fallen into disfavor due to the failure of wild-type p53 gene therapy, despite the fact that p53 mutations are the most common mutations in human tumor cells. Preliminary data from our laboratory shows that fusing p53 with mitochondrial targeting signals (MTS) causes the vast majority of p53 to localize to the mitochondria where, particularly when fused with pro-apoptotic BH3 proteins such as Bad (p53-BH3 constructs), it produces rapid apoptosis in ovarian cancer cell lines. This type of fusion gene therapy has not been attempted before and represents a novel approach for p53-based gene therapy that may resurrect the field. This rapid apoptosis *must* be targeted only to cancer cells so as to avoid high toxicity issues, however. Therefore, ***the focus of my research is to utilize and develop a cancer-specific promoter that will work in concert with our lab's mitochondrially-targeted p53 gene therapy constructs for treatment of ovarian cancer***, the most lethal gynecologic malignancy. Promoters derived from the human telomerase reverse transcriptase (hTERT) promoter as well as a hTERT-CMV promoter fusion (hTC), which appear to grant specificity of gene expression constructs to cancer cells, will be tested with our construct, as well as two additional cancer-specific promoters—RAS-related nuclear protein promoter (ran) and breast cancer metastasis suppressor 1 promoter (brms1)—with a goal to achieve cancer specificity while maintaining high expression levels of our gene constructs. Preliminary results show that, when measured using flow cytometry to determine the % cells expressing GFP under the promoter, a particular segment of hTERT, -279 to +5 relative to the transcription start site (279/5), has the highest activity in Skov3 and Ovcar3 ovarian cancer cell lines (70% and 35%, respectively) compared to other hTERT only promoters. 279/5 is also cancer specific, with a normal cell line (BJ) showing only 11% activity of the 279/5 promoter. The hTC fusion promoter grants higher expression than 279/5 (about 80% in Skov3 cells) but is not as cancer specific as 279/5 (about 20% of normal BJ cells express GFP under hTC. After further testing in

Skov3, Ovcar3, Kuramochi, and ID8 ovarian carcinoma cell lines as well as the BJ normal cell line, the best promoter candidate—highest expression levels with cancer specificity—will be cloned into our lab's top p53-BH3 gene therapy construct and delivered with a CD-PEG-RGD polymer coated adenovirus (low immunogenicity and some cancer specificity) into a syngeneic mouse model of ovarian cancer that closely mimics human disease (developed by our collaborator Dr. Janat-Amsbury, MD/PhD, OB/GYN). If successful, the eventual goal is to produce a new therapy for ovarian cancer, a highly heterogeneous disease with no current effective cure, with elimination or reduction of toxicity compared to traditional cancer therapeutics. Additionally, this "sledgehammer" approach could potentially be applied to many different cancers, regardless of disease heterogeneity, to make a significant impact in the lives of many cancer patients.

596. Design and Assembly of Elastin-Like Polypeptide Nanoparticles for Treatment of Non Muscle Invasive Bladder Cancer

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Bladder cancer will affect an estimated 80,000 people this year in the US alone, making it the fourth most prevalent cancer in men. While surgical removal is generally employed, a 70% reoccurrence is seen within a year. Months to years of maintenance therapy is routinely employed to reduce reappearance. Intravesical injection of Bacillus Calmette-Guerin (BCG) is the primary maintenance therapy for non-muscle invasive bladder cancer. It is believed BCG works as an immunotherapy by activating the local immune response, but BCG-refractory cancer has been increasingly encountered, which has significantly decreased its success rate. Due to limited mechanistic understanding improvements on BCG are difficult, however the treatment of bladder cancer via immune system activation shows potential. Immunostimulatory nucleic acids, such as CpG oligodeoxynucleotides, have shown promise as immunotherapeutic modulators and can potentially be used to supplement the limitations of BCG therapy. Unfortunately, the nuclease instability and low cellular uptake of nucleic acids requires the use of delivery vehicles to enhance efficacy. Charge neutralization of nucleic acids with cationic lipids or synthetic polymers has been a widely used approach for enhancing nucleic acid uptake and efficacy for in vitro applications, unfortunately excess positive charge often leads to cytotoxic responses and nonspecific cell association. To obviate some of these limitations, we sought to use a modular approach wherein a secondary coating, designed to confer cellular targeting and stability until the complex is internalized where it undergoes programmed disassembly, has been developed based on Elastin-Like Polypeptide fusion proteins. Precise chemical modification of the fusion protein and its characterization has been performed on three constructs based on a triblock copolymer architecture which will allow for self-assembly. Nucleic acid polycation complexes have been prepared, and their properties with CpG oligonucleotide sequences and plasmid DNA were evaluated. Studies designed to explore their size, stability, toxicity, and binding to MB49 bladder tumor cells will be reported.

597. Development of a Tumor Specific Promoter for the Treatment of Glioblastoma Multiforme

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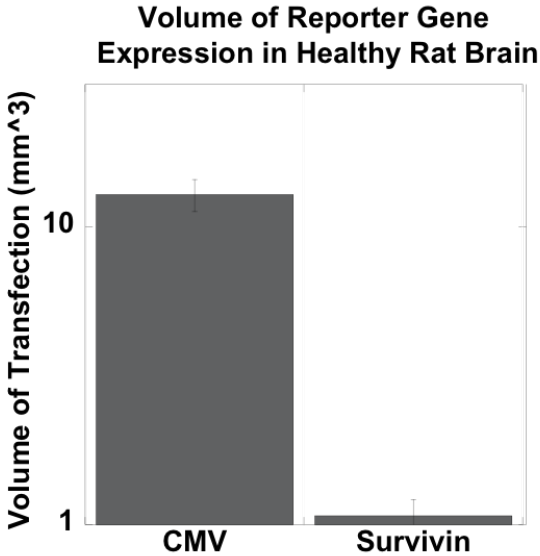
The success of gene therapy for the treatment of glioblastoma multiforme (GBM) has grown tremendously in the last decade; however, its effectiveness has been limited due to the lack of widespread distribution and tumor specificity of therapeutic transgene expression. We have recently shown that sub-120nm particles that are densely coated with PEG, resulting in a net neutral charge, can rapidly diffuse through the brain parenchyma. This DNA-Brain Penetrating Nanoparticles (BPN) have shown broad distribution in the brain parenchyma when compared to the conventional DNA nanoparticles. Similar to clinically used chemotherapeutics, most gene therapies are unable to specifically target tumor regions and can cause toxicity to healthy tissue. To address this critical issue, we have schemed a two-pronged strategy, namely brain-penetrating nanoparticle and tumor-specific promoter. Our hypothesis is that a combination of these approaches would allow for widespread yet cancer-selective delivery of therapeutic nucleic acids, thereby leading to highly effective and safe gene therapy of GBM. To test various tumor-specific promoters (hTERT, HMGB2 and Survivin), *in vitro* screening was conducted in various human, rodent GBM as well as healthy brain cell lines to determine specific tumor expression of fluorescent proteins. After thorough screening *in vitro,* using our lead DNA-BPN, our lead promoter was compared to the conventional CMV promoter to show specificity in healthy rodent brain. When administered in the healthy rat brain, the tumor-specific promoter driven plasmid showed minimal transfection in the brain when compared to the conventional CMV promoter. The use of DNA-BPN in conjunction with the tumor-specific promoter can improve cancer-specific and safe delivery of gene therapy for the treatment of GBM.



598. Delivery of DNA Encoding RNA Therapeutics to Alter the Expression of Oncogenic Transcripts in Glioblastoma

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Glioblastoma multiforme (GBM) is an incurable and aggressive type of brain tumor. It is the most common central nervous system (CNS) malignancy with a median survival of only 14 months. It is characterized by increased activation of one or more tyrosine kinase receptors, particularly epidermal growth factor receptor (EGFR). This receptor is dysregulated in about 60% of GBM tumors. EGFR amplification, over-expression and constitutive activation leads to uncontrolled growth and proliferation of GBM. Although a great deal is known about the aberrant biology exhibited by EGFR-activated GBM, the application of therapies against the biologic processes is limited by the blood-brain barrier, which restricts systemically administered therapies from reaching the brain. Although anti-sense RNAs and small interfering RNAs can be used to target and silence gene expression, exogenously expressed RNAs are susceptible to extracellular nucleases as well as activation of cellular immunity against foreign nucleic acids. To bypass these degradatory mechanisms, we take advantage of a natural noncoding RNA gene architecture and the miRNA expression pathway along with an anti-sense targeted approach to alter EGFR expression. In addition, we make use of a polycistronic delivery system to express RNAs targeting splicing and alternative poly-A signal /G-rich elements of the EGFR transcript. DNA delivery vectors were transfected into human GBM cell lines. Results show that our vectors were expressed at high levels with subsequent reduction in full-length EGFR mRNA expression and concomitant activation of alternative therapeutic isoforms. Current strategies include using the polycistronic delivery mechanism to target additional oncogenic transcripts and adapting to a mouse model of GBM.

599. Low Volume Production of Nanoparticles That Are Effective Transfection Systems in iPSC-Derived Cells, Immune Cells and Other Primary Cell Cultures

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The need for drug delivery and transfection systems is obvious to molecular biologists, disease researchers and scientists in early discovery labs of the pharmaceutical industry. Nanoparticles that serve as these systems must have the potential to be manufactured at sufficiently low volumes to support the genetic manipulation of cells and to conserve costly genetic payloads. Currently, there are no accurate and reproducible production methods to meet the needs of these researchers. The NanoAssemblr™ Spark™ uses a proprietary microfluidics technology for the controlled and reproducible manufacturing of nanoparticles in small volumes (25 - 250 uL). Using a lipid delivery system, that is well described in the literature for the delivery of siRNA, mRNA and plasmid, we have shown the production of lipid nanoparticles (LNPs) that are 60 - 130 nm in size with a polydispersity index (PDI) below 0.2. We have manufactured mRNA-containing LNPs with over 80% encapsulation of payload and shown the effect of various formulation parameters on nanoparticle characteristics. The formulation parameters analyzed included PEG content, N/P ratio, and choice of the cationic/ionizable lipid. mRNA and plasmid LNPs made on the NanoAssemblr™ Spark™ were applied to cells in culture to assess biological activity. Induced pluripotent stem cell (iPSC)-derived neurons were used as neuroscientists are turning to this *in vitro* model to identify potential therapeutic strategies against neurodegenerative diseases. Oligonucleotide-based therapies require the efficient delivery of nucleic acid payloads to these cells, while minimizing cytotoxicity and changes in neuronal physiology. iPSC-derived neurons and iPSC-derived neural progenitor cells (NPCs) were treated with mRNA and plasmid LNPs that were made on the The NanoAssemblr™ Spark™. In this proof-of-concept work, we demonstrate the successful delivery of plasmid LNPs to iPSC-derived NPCs with minimal impact on downstream differentiation into cortical neurons. Over the course of 21 days, these NPCs differentiated into cortical neurons with similar morphological characteristics, such as neurite length, relative to untreated controls. Most critically, this plasmid LNPs treatment had a relatively insignificant impact on neurite length and overall neuron viability. We also show the efficient transfection of mature iPSC-derived cortical neurons using plasmid LNPs. The NanoAssemblr™ Spark™ is ideal for screening novel compositions of nanoparticles that use valuable active pharmaceutical ingredients. This nanoparticle screening platform allows for the rapid advancement of delivery systems that support the development of efficient transfection technologies and the next generation of therapeutics.

RNA Virus Vectors

600. Reactivation of Fetal Hemoglobin in Erythroid Progeny of Bone Marrow CdD34$^+$ Cells from Patients with Beta-Thalassemia Major by Lentivirus-Mediated Expression of IGF2BP1

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The benefits of fetal hemoglobin (HbF) in reducing the severity of β-thalassemia and sickle cell disease has fueled a long-standing interest in identification of factors controlling the switch from fetal to adult hemoglobin (HbA) production. Previously, insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) was shown to be a positive regulator of HbF expression [de Vasconcellos et al., PNAS, 2017]. We independently identified IGF2BP1 as a fetal-specific factor by comparing microarray data for erythroblasts derived from CD34$^+$ cells of fetal liver (FL) which express high levels of HbF versus adult bone marrow (BM) that do not. IGF2BP1 expression in FL and BM

erythroblasts was confirmed by RT-qPCR and compared to BCL11A and LRF/ZBTB7A, two repressors of fetal γ-globin gene expression. IGF2BP1 was highly expressed in FL erythroid cells (42±19% of RNaseP internal control) but at background levels in adult BM-derived cells (0.01±0.01%); a 4200-fold change. Alternatively, LRF/ZBTA7A was detected at similar levels (1.0±0.4% FL vs. 1.7±0.8% BM) and BCL11A only modestly increased (0.2±0.02% FL vs. 1.6±0.3% BM). Thus, IGF2BP1 undergoes a transcriptional switch consistent with the transition from HbF to HbA production. Function of IGF2BP1 was tested by transducing $CD34^+$ cells from health adult donors with lentivirus encoding for bicistronic expression of IGF2BP1 and puromycin under transcriptional control of the erythroid-specific α-Spectrin promoter. Compared to controls, IGF2BP1 expression increased γ-globin mRNA (12.5±6.7-fold increase) and suppressed β-globin mRNA (2.5±0.4-fold decrease) resulting in a 10-fold increase in γ-globin to total globin levels (γ/γ+β=0.07 control; 0.72 IGF2BP1, p=0.003). High performance liquid chromatography (HPLC) showed a robust increase in HbF for erythroid cells with IGF2BP1 expression (IGF2BP1: 48±10%; control: 3.4±2.8%; p=0.002). Of the potential mechanisms, western blot revealed diminished levels of BCL11A, although mRNA was essentially unchanged, suggesting translational repression. While successful, these studies identified challenges to production of high-titer lentivirus which we attributed to binding of IGF2BP1 to the internal ribosome entry site (ires) used to couple expression of IGF2BP1 and the puromycin resistance gene. To address this, IGF2BP1 was fused to puromycin or ZsGreen fluorescent protein using the 2A peptide and placed under control of the constitutive spleen focus forming virus (SFFV) promoter or α-Spectrin promoter. The 2A fusion constructs significantly improved vector titer (up to 20-fold). Erythroblasts derived from transduced $CD34^+$ cells of healthy adult donors demonstrated increased γ-globin and concordant reduction in β-globin mRNAs (γ/γ+β ratio=0.13 control; 0.81 α-Spectrin-IGF2BP1; 0.64 SFFV-IGF2BP1) and mean levels of HbF were 50% of total hemoglobin. Independent tests were performed using BM $CD34^+$ cells from patients with β-thalassemia major. While these patients exhibited a higher baseline level of γ-globin to total globin mRNA (mean 0.61), IGF2BP1 overexpression shifted this ratio to 0.92 (α-Spectrin) and 0.99 (SFFV). Flow cytometry analysis demonstrated that vector-mediated expression of IGF2BP1 was within the physiological range of erythroblasts derived from FL $CD34^+$ cells. Similar to healthy donors, western blot confirmed translational inhibition of BCL11A. The resulting levels of HbF ranged from 47% to 49%, which increased total Hb content per cell to 50% of normal, which is Thal trait. We conclude that expression of IGF2BP1 has the potential to maximize HbF expression in patients with severe β-thalassemia or sickle cell disease.

601. Intraosseous Delivery of Lentiviral Virus Acts as a Therapeutic Strategy for *In Vivo* Gene Therapy of Hemophilia

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In our previous studies, we have proven that lentiviral vectors (LVs) delivered by intraosseous (IO) infusion at a precisely controlled speed can efficiently transduce bone marrow hematopoietic stem cells (HSCs) in mice. IO infusion of LV carrying a human FVIII/N6 transgene driven by a platelet-specific Gp1bα promoter into hemophilia A (HemA) mice produced FVIII stored in platelet α-granules. These platelet FVIII partially corrected the bleeding phenotype over five months in HemA mice with or without pre-existing anti-FVIII inhibitors. In the current study, we applied this promising strategy, IO delivery of LVs, in humanized NSG mice to establish a translational research model for *in vivo* gene therapy of hemophilia. Firstly, we evaluated if high level transgene expression could be achieved in human megakaryocytes (Megs). Human $CD34^+$ cells were transduced with Cocal-MND-GFP-LV (M-GFP-LV, MOI = 25) or Cocal-Gp1bα-GFP-LV (G-GFP-LV, MOI = 25) and then cultured with Megakaryocyte Expansion Supplement (MES). 14 days after LV transduction, high level of GFP expression (36%) was detected in both M-GFP-LV transduced Megs ($CD41^+$ cells) and non-Megs ($CD41^-$ cells). Furthermore, in G-GFP-LV-transduced human cells, GFP specifically expressed in Megs, but not in other types of cells derived from HSCs. Next, FVIII specific expression in human Megs was evaluated in Cocal-GP1bα-F8/N6-LV (G-F8/N6-LV) transduced human $CD34^+$ cells. We found that FVIII expression level in G-F8/N6-LV transduced human cells increased from 7% to 28% when the LV doses increased from MOI = 10 to 50 on day 21 after LV transduction. These results indicated that platelet-specific promoter, Gp1bα, could specifically guide high level transgene expression in human Megs. Secondly, we investigated transgene expression in humanized NSG mice following IO delivery of LVs. Humanized NSG mice were created by I.V. infusion of 1×10^6 human $CD34^+$ cells into 6-week female NSG mice. After 8 weeks, G-GFP-LV, G-F8/N6-LV and Cocal-GP1bα-F8X10-LV (G-F8X10-LV) were delivered into the humanized mice by IO infusion and transgene (GFP or human FVIII) expression was evaluated in human platelets. However, human platelets in blood of humanized mice rapidly decreased to undetectable levels following human cells engraftment probably due to its recognition and destruction by murine macrophage cells. In order to examine transgene expression in human platelets, the mice were given a series of clodronate liposome treatments (CLT) (0.7 mg/animal, Day 0, 2, 7, 13), which was reported to transiently deplete murine macrophage cells. CLT treatment induced recovery of human platelets from an undetectable level to the level comparable to the percentage of engrafted human cells in total PBMCs. In addition, similar level of GFP expression was detected in both human and murine platelets of CLT + G-GFP-LV treated mice. Most importantly, higher platelet FVIII level was found in CLT + G-F8/N6-LV or G-F8X10-LV treated mice than G-F8/N6-LV or G-F8X10-LV only treated mice. Furthermore, over 10% human cells expressed FVIII when human $CD34^+$ cells were isolated from the G-F8/N6-LV-treated humanized mice and cultured with MES for 6 days. Next, LV integration sites in isolated human cells were examined by LAM-PCR. Taken together, IO infusion of LVs could be a valuable strategy for *in vivo* gene therapy of hemophilia A.

602. Novel Regulation of MGMTP140K Gene Modified CD34+ Cells Improves Engraftment Without Compromising Chemoprotection or in Vivo Selection

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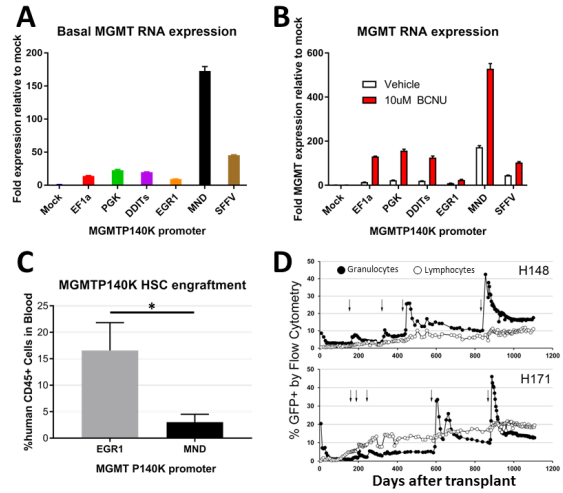
Drug resistance gene therapy with mutant MGMT (P140K)-modified hematopoietic CD34+ cells circumvented myelosuppression associated with chemotherapy in a phase I clinical trial in glioblastoma patients[1]. While studies in nonhuman primates demonstrated both chemoprotection and chemoselection in vivo, in human studies engraftment and persistence of MND promoter-regulated P140K gene modified cells was transient and dependent upon chemoselective pressure. Here, we demonstrate that low-level P140K expression in CD34+ cells can improve engraftment, chemoselection and protection from chemotherapy-induced cytopenia. P140K transgenes were cloned into the same self-inactivating lentivirus with different human or viral promoters: EF1a, PGK, EGR1, DDITs, MND or SFFV. EGR1 and DDITs were identified by a screen of KG1a cells as activated following chemotherapy. Transduction of human CD34+ cells with equivalent MOI of each LV vector demonstrated P140K transcript and protein expression with up to a 170-fold difference compared to untransduced cells (Figure 1A). MND and SFFV promoters drove expression at 50-170 fold, while low-level constitutive promoters PGK and Ef1α had a 15-20 fold increase in expression. EGR1 and DDITs exhibited 10-20 fold increase in expression compared to untransduced CD34+ cells. To determine whether P140K expression was induced by chemotherapy, we treated transduced cells with 10μM BCNU and measured MGMT transcript (Figure 1B). Regardless of promoter, MGMT RNA increased after chemotherapy and protected from DNA damage in vitro. To determine whether P140K expression levels impacted engraftment, transduced human CD34+ cells were infused into immunodeficient mice. We compared EGR1 and MND because these two promoters covered the largest range of MGMT expression (10 to 170 fold increased). Here we observed a three-fold significant increase (P=0.035) in the level of engraftment in favor of CD34+ cells transduced with EGR1-P140K expression (Figure 1C). To assess hematopoietic reconstitution, chemoselection and chemoprotection in the context of autologous, myeloablative transplantation, we utilized the canine model. We infused 1.94-2.3e6 CD34+ cells per Kg transduced with EGR1-P140K. After O6BG and BCNU chemotherapy, gene modified leukocytes increased from 1-3% to 17-18% (Figure 1D). These data demonstrate the utility of optimized P140K expression in clinical gene therapy applications.

1. Adair, J. E. *et al. J. Clin. Invest.* **124,** 4082-4092 (2014).

Figure 1: Low P140K expression improves engraftment while providing chemoprotection and selection. A: qPCR measuring MGMT mRNA transcripts. CD34 cells from 3 healthy human donors were transduced with equal MOIs of lentivirus expressing P140K. B: qPCR measuring increase in expression of MGMT. LV transduced CD34 cells were treated with 10μM BCNU or vehicle for 8h. C: Human HSC engraftment is reduced in MND driven P140K transduced HSCs. Human CD34 cells were transduced with equal MOI LV P140K vector driven by EGR1 or MND. After transduction, cells were injected into neonate NOD scid gamma mice (1e6 cells / mouse, n=4). Human CD45 cells were measured 10 weeks post injection. D: Chemoselection of EGR1-P140K LV gene modified cells in canines. Dogs were injected with CD34 cells transduced with EGR1-P140K-PGK-GFP lentiviral vector. Arrows indicate O6BG/BCNU administration in vivo.



603. Development of Transmission-Deficient Borna Disease Virus Vector with Enhanced Transduction Efficiency in Primary and Stem Cells

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Introduction A negative strand RNA virus, Borna disease virus (BoDV), establishes persistent infection in the cell nucleus without cytopathic effect. This virus can uniquely maintain intranuclear infection by interacting its replication complex vRNP with host chromosome. These features make BoDV ideal for RNA virus vector that enables long-term expression of a foreign gene without disruption of host genome. In a previous study, we also reported that BoDV vector could deliver a foreign gene for a long-term in several stem cells, including iPS cells without affecting their pluripotency. Although these results suggest that BoDV vector would be a potent tool for gene therapy and regenerative medicine, current challenge associated with this vector is its low viral titer. In this study, we aimed to generate BoDV vector with improved viral titer using glycoproteins (G) from different genotypes of *Bornavirus*.

Results and Discussion We generated transmission-deficient BoDV vectors, which were transiently enveloped by G protein derived from 9 different genotypes within the genus *Bornavirus* and evaluated their transduction efficiencies in cultured cells. Among 9 different G proteins examined, BoDV vector pseudotyped with G protein from Canary bornavirus 1 (CnBV-1) showed the highest viral titer in several established cell lines. Importantly, BoDV vector with CnBV-1 G protein significantly increased the transduction efficiency of primary glial and human iPS cells, compared to the original BoDV vector. Furthermore,

by comparing chimeric G proteins of two closely related viruses CnBV-1 and CnBV-2, we determined the membrane transport signal of G protein to be important for increasing the viral titer of CnBV-1. Our study indicates that BoDV vector enveloped with CnBV-1 G protein could be a new platform for gene therapy. We are currently assessing feasibility of CnBV-1 G enveloped BoDV vector for gene therapy for neurodegenerative diseases.

604. Potential Utility of Mesenchymal Stem Cells as Retroviral Replicating Vector Delivery Vehicles for Gene Therapy of Cancer

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Retroviral replicating vectors (RRVs) have shown promising clinical results for gene therapy of cancer, and are currently being evaluated in an international Phase III trial for recurrent high-grade glioma. Previously we have developed two different RRVs, derived from amphotropic murine leukemia virus (AMLV) and gibbon ape leukemia virus (GALV) respectively, which utilize different cellular receptors for viral entry. Both RRVs have been shown to achieve tumor-selective replication, efficient tumor transduction and enhanced therapeutic benefit in a wide variety of cancer models. Since retrovirus-based vectors are produced only a low titer in vitro and easily inactivated by complement in blood, direct injection into local tumor tissue has been the preferred route of administration. However, direct intratumoral injection is not feasible in certain clinical situations, for example when tumors are anatomically difficult to access or metastatic disease with multiple and/or disseminated tumors, hence it is relevant to develop alternative methods for efficient delivery of RRV to tumor sites in vivo. Accordingly, in the present study, we evaluated mesenchymal stem cell (MSC)-based RRV producer cells as 'RRV carrier vehicles' which preferentially accumulate and engraft at tumor sites. Here we tested human MSCs derived from adipose tissue (HMSC-ad), bone marrow (HMSC-bm) and umbilical cord (HUMSC). All MSCs were infected efficiently with both AMLV- and GALV- RRVs, but produce virus progeny less efficiently than tumor cells. We then assessed tumor cell tropism of the MSCs, using a Transwell plate in vitro migration assay with mesothelioma cells as the target. All three types of the MSCs showed significant trans-migration toward the mesothelioma cells, although the migration abilities were different each other. Furthermore, when co-cultured, RRVs were found to be transmitted efficiently from MSCs to mesothelioma cells, thereby achieving high levels of tumor cell transduction. These data indicate the potential utility of MSC-mediated delivery of RRVs for cancer gene therapy.

605. The Composition of Lentiviral Vector 5' Leader Sequences Can Affect Their Therapeutic Efficacy

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Conventional lentiviral (LV) vectors require 20% of the wild-type HIV-1 genome for efficient packaging into viral particles. But we have recently shown this content can be minimized by using LTR1 vector technology, which allows customization of the 5' leader sequence upstream of a transgene cassette and elimination of the HIV-1 'Ψ' packaging sequence. In recent studies, we have been investigating potential mechanisms for the enhanced transgene expression that we have detected from LTR1 vectors *in vivo*.We and others have observed that CpG islands present in the Ψ packaging sequence are methylated more rapidly than CpG islands in the transgene promoter. These observations led to suggestions that Ψ may serve as a *de novo* "methylation center" that initiates spread of DNA methylation to the transgene promoter, resulting in eventual silencing of the expression cassette. Thus, we hypothesized that the CpG content of the LV vector 5' leader could have an important effect on the long-term activity of transgene promoters. To investigate this in the context of LTR1 technology, we constructed LTR1 vectors with either a CpG-rich leader (LTR1.27 = 52% CpG) or a CpG-null leader (LTR1.25 = 0% CpG) and investigated their performance *in vivo*, compared to a conventional LV backbone (CCL = 11% CpG). We packaged CCL, LTR1.25, or LTR1.27 with a therapeutic Factor IX (FIX) transgene cassette and treated Factor IX-deficient mice on the day of birth with intravenous injections of purified vector preparations. Interestingly, we found that, whilst integrated vector copy numbers were detected in the liver in equal numbers, the LTR1.27 vector restored plasma FIX levels to 24% of healthy levels, whereas LTR1.25 restored just 15% levels and CCL restored 13%, suggesting that the CpG-rich LTR1.27 backbone could have a positive effect on liver expression. We investigated a potential mechanism for this effect by transducing murine embryonic p19 cells with either LTR1.27 or LTR1.25 and tracking their rate of silencing *in vitro*. This p19 cell line was chosen as it is known to rapidly methylate and silence vector genomes. This study corroborated findings in the FIX model, as LTR1.27 expression gave significantly more sustained expression in p19 cells *versus* LTR1.25 over a 2-week period (P=0.001). We then investigated the performance of each vector in the brain, packaging a luciferase transgene into either CCL, LTR1.25, or LTR1.27. Titre-matched vectors were intracranially administered to neonatal mice, with bioluminescent output tracked over a 20-day period. However, in this case we found that LTR1.25 matched the expression level of LTR1.27 (P=0.940), but both LTR1 backbones gave greater expression than the conventional CCL vector (P=0.038). These results suggest that, although LTR1 vectors provide enhanced levels of transgene expression *versus* existing LV technology *in vivo*,

this effect is not exclusively due to the density of CpG islands in their 5' leader sequences. Thus, we are actively investigating further mechanisms to explain this property.

606. Characterization of BoDV Vector Encoding Thymidine Kinase for Cancer Gene Therapy

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Borna disease virus (BoDV) is an RNA virus that infects a wide range of vertebrates. An intriguing characteristic of BoDV is its ability to establish a long-lasting persistent infection in the nucleus without integrating into the host genome. In our previous study, we exploited unique biological characteristics of BoDV and developed a non-integrating vector system capable of sustained transgene expression. BoDV vector has been shown to efficiently transduce human induced pluripotent stem cells (hiPSCs) without disrupting their pluripotency. Recently, we found that glioblastoma and neuroblastoma cell lines are highly susceptible to infection by BoDV vector, suggesting that BoDV vector might have potential as a vector for cancer gene therapy applications. In this study, we developed BoDV vector encoding thymidine kinase (TK) and conducted in vitro studies to evaluate anti-tumor effects of BoDV TK vector. Herpes simplex virus TK was inserted in BoDV vector containing GFP as a selection marker. The recombinant vector was rescued using reverse genetics system of BoDV. To generate carrier cells for targeted delivery of BoDV vector to cancer cells, hiPSCs were transduced with BoDV TK vector, and differentiated into neural stem cells (iNSC)s. Culturing hiPSCs transduced with BoDV TK vector for one week in neural induction medium readily differentiated these cells into iNSCs. Expression levels of NSC markers Nestin and Pax6 were similar in both mock transduced and BoDV TK vector transduced cells, supporting previous finding that iPSCs transduced with BoDV vector maintain pluripotency. Migration of generated iNSCs to cancer cells was confirmed by modified boyden chamber assay. Ongoing experiments evaluating anti-tumor effects of BoDV TK iNSCs and ganciclovir (GCV) against glioblastoma and neuroblastoma will be presented.

607. Optimization of the Production and Concentration of Pre-Clinical Grade Jaagsiekte Sheep Retrovirus (Jsrv) Env-Pseudotyped Lentiviral Vectors for Lung Gene Delivery

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Lung gene therapy requires stable expression of delivered genes as well as efficient transduction of the slow-replicating epithelia. Lentiviral vectors (LV) have the coding, expression and transducing capacity required for gene therapy. The recently gene therapy tailored small ruminant betaretroviral envelope protein from JSRV (Jenv) is especially fitting for LV-mediated lung gene therapy because of the virus's inherent tropism for the lung and the demonstrated ability of Jenv to efficiently pseudotype LVs. However, studies detailing how to effectively concentrate Jenv-pseudotyped LV are needed, particularly because the envelope protein can influence concentration of LVs. The aim of the current study was to establish a method that would yield high titer, preclinical grade Jenv-pseudotyped LV suitable for use in vivo in mice as well as to establish a method for scaling up production of Jenv-pseudotyped LV. Here we report that ultracentrifugation drastically reduced the yield of Jenv LV produced in contrast to vesicular-stomatitis-virus-G protein (VSVg) and Ebola virus glycoprotein (EBOV GP) pseudotyped LV. Conversely, PEG precipitation and tangential flow filtration (TFF) were found to be suitable methods for concentrating Jenv-pseudotyped LV. We also highlight the importance of pressure in TFF to increase the recovery of LV in general, and Jenv LV in particular. We also optimized a protocol for the use of precision-cut lung tissue slices to test gene therapy vectors and used these tissue slices to evaluate the efficiency of transduction by Jenv-pseudotyped LV. Our work with lung tissue slices showed that the VSVg LV was less efficient in transducing murine than ovine tissue, and this was found to be the opposite for EBOV GP LV. As predicted, Jenv LV was the most efficient vector at transducing ovine tissue. Our results also showcase the importance of choosing an appropriate lung model for titrating and testing the efficacy of LVs for gene delivery purposes, and that precision-cut lung tissue slices offer an attractive method for the evaluating of LV transduction prior to in vivo work.

608. Developing Strategies to Evade Interferon Response during Gene Therapy with Viral Vectors

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Introduction: Activation of innate immune responses is a first barrier during gene therapy with viral vectors. Previous studies have demonstrated a significant role of interferons (IFN) in limiting the therapeutic efficacy of viral vector mediated gene therapies suggesting strategies to inhibit or evade IFN signaling may increase the efficacy of gene therapy with viral vectors. Among human RNA viruses, hepatitis C virus (HCV) has evolved with multiple mechanisms to evade host innate and adaptive immune responses to establish viral persistence. Previous studies have found that non-structural protein 5A (NS5A) of HCV inhibits IFN signaling; however, the NS5A motif(s) required for this are not known. Here we studied lentiviral vectors expressing various NS5A motifs for their ability to inhibit IFN signaling in human cell lines. **Methods**: Lentiviral vectors expressing various motifs of HCV NS5A were produced in HEK 293T cells. Lentiviral vector expressing GFP was used as a control. These vectors were used to transiently or stably express NS5A in HEK293T cells. IFN signaling was assessed by measuring tetherin/ BST-2/ CD317 (interferon-stimulated gene) expression by flow cytometry following IFN stimulation. Deletion mutagenesis of NS5A was performed to identify motifs within NS5A required for inhibiting IFN signaling.

Results: HEK 293T cells stably expressing full length NS5A from HCV genotype 2a significantly inhibited IFN signaling compared to control cells stably expressing GFP. Deletion mutagenesis studies identified a region within the C-terminus of NS5A designated as low complexity sequence II (LCS-II) potentially required for inhibition of IFN signaling. HEK293T cells stably expressing LCS-II from HCV genotype 1b also showed diminished IFN signaling compared to controls, though to a lesser extent than full length NS5A. **Conclusions:** These data demonstrate that HCV NS5A is sufficient to attenuate IFN signaling in human 293T cell line. Expression of the LCS-II domain of NS5A also inhibits IFN signaling but to a lesser extent compared to full-length. Current studies are underway to further characterize NS5A for its ability to inhibit IFN signaling, and assessing the ability of this protein to inhibit IFN signaling in clinically relevant cell types such as macrophages. Since NS5A expressing lentiviral vectors decrease IFN signaling, incorporating this NS5A motif in the design of viral vectors may be an effective strategy to reduce IFN response during gene therapy.

609. Analysis of Lentivirus Integration Site Distributions in CTL019 Immunotherapy

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Tisagenlecleucel (CTL019) is an adoptive cancer immunocellular therapy which involves the reprogramming of autologous T cells with a transgene encoding a chimeric antigen receptor (CAR), which allows these T cells to target and destroy CD19 positive malignant cells. This report presents the investigation of genomic insertion sites by the lentiviral vector used for delivery, and focuses on integration site distributions and implications for safety. Specifically, we investigated whether the CTL019 lentiviral vector might integrate into sites of concern preferentially. Adverse events have taken place in human gene therapy in which gammaretroviral vectors integrated near the 5' ends of cancer-associated genes such as LMO2, CCND2, and MECOM. Cells proliferated, accumulated more genetic lesions, and eventually evolved to frank leukemia. Clonal expansions without clinical consequences have been observed for lentiviral vectors in HMGA2. Here fourteen DNA samples were studied from experimental infections of T-cells with the CTL019 lentiviral vector from 12 cancer patients (6 ALL and 6 DLBCL) or 2 healthy controls. Informed Consent Forms were obtained for all clinical samples analyzed as part of the clinical protocols CCTL019B2202 and CCTL019C2201, as well as from healthy volunteers obtained from Hemacare (Van Nuys, CA, United States). Integration site sequences were determined and analyzed using the INSPIIRED pipeline (integration site pipeline for paired end reads). All samples were highly polyclonal, either by traditional (S. chao, Gini or Shannon indexes) or recently developed UC50. Integration site distributions were as expected for lentiviral integration in human T-cells, showing integration in gene rich regions, within transcription units, and near epigenetic marks associated with active transcription. There was no indication of preferential integration near genes of concern, or preferential outgrowth of cells harboring integration events near genes of concern in the pre-infusion cell expansion.

Synthetic/Molecular Conjugates and Physical Methods for Delivery of Gene Therapeutics I

610. Engineering of DNA-Encoded PCSK9 Monoclonal Antibodies as Novel Lipid-Lowering Therapeutics

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Cardiovascular disease is the leading cause of death worldwide. Elevated LDL-C is one of the major contributors to cardiovascular heart disease. Statins have been found to be very effective in lowering LDL-C levels, however serious adverse side-effects have been reported, resulting in some cases to discontinuation of the treatment. Alternative treatment strategies are required for patients deemed statin-intolerant. One of the most promising alternatives to statins are PCSK9 inhibitors. Protein-based monoclonal antibodies (mAbs) against PCSK9 have shown great efficacy in LDL-C reduction. Unfortunately, the high cost of protein-based mAbs is one of the major constraints in clinical management of at-risk cardiovascular patients. Here we developed an anti-PCSK9 DNA-encoded monoclonal antibody (DMAb) as a suitable alternative to protein-based lipid-lowering therapeutics. We demonstrate successful suppression of PCSK9 in vivo and elevation of liver LDLR levels in DMAb-treated mice. Single intramuscular administration of PCSK9 DMAb plasmid DNA was able to lead to significant reduction in non-HDL and total cholesterol in mice. We achieved a substantial 28.6% decrease in non-HDL and 10.3% decrease in total cholesterol by day 7 in wild type mice with mouse PCSK9 DMAb. Remarkably, repeat dose administration of mouse PCSK9 DMAb led to increasing expression kinetics, with DMAb levels of around 7.5ug/ml at day 62 and a reduction of around 20.9% in non-HDL. DNA-encoded monoclonal antibody-based therapeutics could provide a novel, more cost-effective approach to reducing LDL-C, overcoming the challenges associated with recombinant protein-based monoclonal antibody therapeutics. The DMAb technology could considerably advance the field of immunotherapeutics for treatment of atherosclerosis and numerous other diseases.

611. pmIL12 Gene Electrotransfer Generates an Effective Immune Response in Established Mouse B16F10 Melanoma

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Melanoma is a deadly form of skin cancer. Due to aggressiveness and metastatic spread, survival of patients diagnosed late is fairly low. Immune-based therapies have shown promise in the treatment of melanoma patients but are limited by low overall complete response rates. We previously have shown that delivery of a plasmid encoding IL-12 would result in complete regression and stimulation of a protective immune response in the B16F10 mouse melanoma model. Optimum electrotransfer conditions (voltage, number and duration of impulses, separation of caliper electrodes) were established to manipulate expression levels of the transgene. So, through manipulation of GET parameters the onset, level, and duration of protein expression of IL-12 can be controlled, resulting in meaningful therapeutic effects in melanoma without dose-dependent IL-12 toxicities. In the current study, we sought to examine the host immune response to melanoma and to delineate which cells or cytokines may be involved. Understanding cell interactions and activation patterns could have important clinical ramifications. Using a B16F10 mouse melanoma model, we have shown that melanoma exhibits unique immune cell composition within the tumor microenvironment after intratumoral injection of pmIL12 with gene eletrotransfer (GET). The total number of memory immune cells was markedly increased in pmIL12 GET melanoma groups compared with control group, which was validated using flow cytometry to analyze peripheral blood mononuclear cells. We further identified key differences in immune cell content using immunohistochemistry. pmIL12 GET displayed significant regulation of multiple immune cell types, including CD8+ cells, regulatory T cells and myeloid cells, which were induced to mount a CD8+ immune response. Taken together, these findings suggest a basic understanding of the sequence of immune activity following pmIL12 GET and demonstrate that adjuvant immnuotherapy shapes the host immune response to cancer at different time points.

612. Activation of Poly(Amine-Co-Ester) Terpolymers for Potent and Safe mRNA Delivery

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DNA-based gene therapies are either inefficient or associated with long-term safety risks; further, they generally lack an ability to control protein expression. mRNA-based therapies potentially address these concerns, but require an efficient delivery method that is also safe enough for chronic dosing. We previously described the use of biodegradable poly(amine-*co*-ester) (PACE) terpolymers for efficient DNA delivery. In the present work, we evaluate the potential of PACE to deliver mRNA. We observe that the transfection efficiency of PACE is highly dependent on its molecular weight and end group compositions. Therefore, we developed a reproducible method to simultaneously vary these two factors by a process we called activation. The activated PACE (aPACE) presents superior mRNA delivery properties compared to regular PACE, with up to a 10^6-fold-increase of mRNA transfection efficiency on HEK293 cells. Moreover, when aPACE was used to deliver mRNA coding for erythropoietin (EPO) *in vivo*, it produced high levels of EPO in the blood for up to 48 h, without inducing systemic toxicity. This polymer constitutes a new delivery vehicle for mRNA-based treatments that provides safe yet potent protein production.

613. ZBP1/DAI Protein Binds Plasmid DNA Directly after Electroporation into C2C12 Mouse Myoblasts

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During the last two decades, numerous works from different groups proved the importance of cytoplasmic nucleic acid sensing in a variety of physiological and pathological processes, including anti-pathogen defense and autoimmunity. Nucleic acid sensors are widely expressed in a variety of cells as a subset of pattern-recognition receptors (PRRs), but the detailed mechanisms of nucleic acid sensing have not been clarified. Our previous studies confirmed the cell type-specific upregulation of PRRs on both the mRNA and protein levels after electroporation of plasmid DNA (pDNA) into the cells. Type I interferon was found to be upregulated as well, which implicates innate immunity pathway activation in response to pDNA entry into the cytoplasm. Still, the sequence of events after pDNA electroporation remains unclear. Using molecular and proteomic techniques, we identified ZBP1/DAI protein as the initial PRR binding pDNA entering the cytoplasm after electroporation and estimated the participation of other PRRs early in this process in mouse myoblast C2C12 cells. Better understanding of the molecular underpinnings of nucleic acid sensing by PRRs may allow us to manipulate the process of innate immunity activation, developing new and improving existing therapies for immunity- and tumor-related diseases.

614. Poly(amine-co-ester) Nanoparticles for the Delivery of Peptide Nucleic Acid Gene Editing Agents to Correct Beta-thalassemia

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Efforts to correct hereditary genomic mutations have advanced with the improvement of genome engineering technologies such as

active nuclease-based platforms including CRISPR/Cas9, and oligo/polynucleotide strategies such as triplex-forming oligonucleotides (TFOs). Programmable RNA-guided Cas9 endonucleases enable efficient editing, but also exhibit high off-target effects. Moreover, *in vivo* delivery of CRISPR/Cas9 remains challenging. These drawbacks have motivated the development of non-nuclease-based platforms, such as peptide nucleic acids (PNAs). These TFOs have a peptide backbone but undergo base pairing with DNA/RNA. PNAs designed to bind to sites in genomic DNA and form PNA/DNA/PNA triplexes can initiate an endogenous DNA repair response mediated by nucleotide excision repair and homology-dependent repair pathways. Further, these molecules are readily encapsulated in polymeric nanoparticle (NP) delivery systems. When PNAs are co-delivered with a single-stranded donor DNA containing the desired sequence correction, site-specific modification of the genome occurs. Blood disorders caused by a single mutation such as β-thalassemia are attractive targets for gene editing. PNA/DNA therapeutics have successfully ameliorated this disease *in vivo* when encapsulated and delivered in FDA-approved poly(lactic-co-glycolic acid) (PLGA) polymeric NPs, mediating significant gene editing of the β-thalassemia-associated IVS2-654 mutation in the bone marrow (BM), and leading to functional improvement with extremely low off-target effects (1). However, important challenges remain. PLGA NPs can be loaded reproducibly with nucleic acids (NAs); an optimal delivery vehicle would have high encapsulation efficiency, efficient transfection activity, and broad control over release kinetics. We have developed a family of **novel poly(amine-co-ester) (PACE) polymers which demonstrate significant promise for *ex vivo* and *in vivo* PNA/DNA delivery.** Promising attributes of PACE NPs are high NA loading, sustained cargo release, and low toxicity (2). PACE polymers are **biocompatible/biodegradable, highly customizable, and mildly cationic, which aids in NA association**. The polymers are synthesized in small batches via enzymatic copolymerization of diesters with amino-substituted diols. Classic PACE has three monomers: a lactone that confers hydrophobicity, an amino-diol that confers a cationic charge, and an ester. Altering feed monomer ratios, particularly the lactone, yields materials with different physical and chemical properties. We synthesized higher lactone content polymers (50-80 mol%), as these form solid NPs using the same emulsion methods as used for PLGA. Preliminary data reveal that PACE NPs are taken up efficiently in primary BM and mouse embryonic fibroblast (MEF) cells (Fig. 1 A, B). **Further, BM cells treated *ex vivo* with PNA/DNA-loaded PACE NPs exhibited increased gene editing of the IVS2-654 mutation compared to PLGA (Fig. 1C).** *In vivo* treatment of thalassemic mice with PNA/DNA-loaded PACE NPs is expected to result in elevated gene correction and disease amelioration.

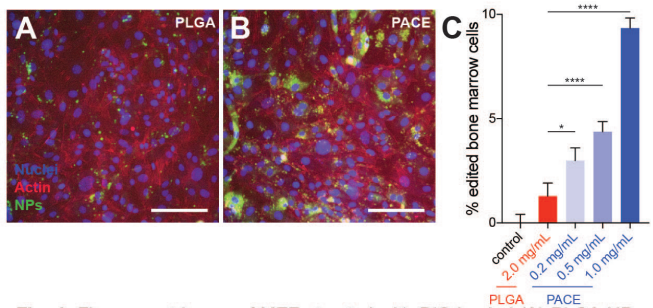

Fig. 1. Fluorescent image of MEFs treated with DiO-loaded (A) PLGA NPs or (B) 60% PDL PACE NPs at 200 μg/mL after 24 h. (C) Quantification of gene editing of the IVS2-654 mutation in BM cells treated with PNA/DNA-loaded PLGA or 60% PDL PACE NPs after 48 h. Scale bars, 50 μm. *p<0.05, ****p<0.0001.

References (1) Bahal R, et al. *Nat Commun*. 2016. (2) Zhou J, et al. *Nat Mater*. 2011.

615. Development of Nanoparticles to Deliver a CRISPR/Cas9 Therapy for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a muscle wasting disease caused by frame-shifting mutations in the *DMD* gene, which encodes dystrophin protein. Recently, we developed and validated a CRISPR/Cas9 platform that can restore the *DMD* reading frame in patient derived hiPSCs *in vitro* and in our humanized mouse model *in vivo*. We next sought to achieve direct, systemic delivery of the platform *in vivo*. Currently, the DMD field is focused on using AAV to deliver nucleic acids to skeletal muscle; however, AAV is not ideal for delivering CRISPR/Cas9, due to adverse issues that include the immune response and an incapacity for repeated delivery. To accomplish systemic delivery of our CRISPR platform, we have developed a polymer-based nanoparticle (NP). The polymer NPs are cationic and interact with nucleic acid via electrostatic interactions to self-assemble into a nanoparticulate structure (NP:DNA). In order to assess NP:DNA trafficking *in* vitro, we delivered FITC labeled NP complexed with Cy3-DNA or with a plasmid expressing a TdTomato reporter in primary murine muscle cells. We found efficient NP:DNA uptake and expression of TdTomato 24 hours post incubation. This result suggests that our NPs can escape the lysosome and release the plasmid cargo and translocate to the nucleus to express the TdTomato reporter. To test the efficacy of our NPs in the context of DMD, we tested our CRISPR platform (NP:CRISPR) *in vitro* on primary muscle cells from our humanized mouse model. Gene-editing was detected as early as 1 week post NP:CRISPR delivery. To examine trafficking of our NPs *in vivo*, we systemically delivered FITC-NP:Cy3-DNA and imaged muscle groups 24 hours post administration. Optical imaging detected a positive Cy3 signal in numerous skeletal muscle groups such as the quadriceps and gastrocnemius. To assess *in vivo* trafficking of our NPs, we sectioned muscles that showed the highest NP:Cy3-DNA uptake

by optical imaging. NP:DNA particles were able to extravasate the bloodstream and enter the muscle, but most were observed to be lodged in the basement membrane of muscle fibers or within the interstitial space between fibers. Some components of the basement membrane are anionic; thus, we hypothesize that our positively charged NPs are interacting with negatively charged proteins in the basal lamina. These studies are proof of principle that NP:CRISPR can be systemically delivered and can home to skeletal muscle *in vivo*. We are currently developing strategies to neutralize the cationic charge of our NPs to improve muscle penetration through the muscle extracellular matrix.

616. Reversed Expression of Aging-Related Molecules by Transfection of Circulatory Biomarkers from Whole Blood and Exosomes

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Molecular changes during aging have been identified to understand the mechanism of aging progress, and also to use determine the status of aging progress. We investigated that changes in miRNA expression in the whole blood and exosomes of mice. The molecules selected as circulatory biomarkers from whole blood and exosomes were tried to induce reversed expression of aging-related molecules in tissues, and proposed to cause systemic reverse of aging in vivo. Through next generation sequencing analysis, we selected 27 differentially expressed miRNAs in whole blood of mice during aging. The most recognized function involved was liver steatosis, a type of non-alcoholic fatty liver disease (NAFLD). Among 27 miRNAs, six were predicted to be involved in NAFLD, miR-16-5p, miR-17-5p, miR-21a-5p, miR-30c-5p, miR-103-3p, and miR-130a-3p. The expression of the genes associated in the network of these miRNAs, Bcl2, Ppara, E2f1, E2f2, Akt, Ccnd1, and Smad2/3, was also altered in the liver of aged mice. Following transfection of these miRNAs into old mice, levels of transfected miRNAs in liver increased, and expression of Mre11a, p16INK4a, and Mtor, reported to be aging-associated molecules, was also reversed in the livers. In exosomes from 12-month-old mice, mmu-miR-126-5p and mmu-miR-466c-5p levels were decreased and mmu-miR-184-3p and mmu-miR-200b-5p levels were increased significantly, compared to those of 3-month-old mice. In the aged tissues injected with young exosomes, mmu-miR-126b-5p levels were reversed in the lungs and liver. Expression changes in aging-associated molecules in young exosome-injected mice were obvious: p16Ink4A, MTOR, and IGF1R were significantly downregulated in the lungs and/or liver of old mice. In addition, telomerase-related genes such as Men1, Mre11a, and Tnks were significantly upregulated in the liver of old mice after injection of young exosomes. Therefore, the identified molecules in whole blood and exosome might induce a reverse-regulation of aging-associated pathways. This study provides preliminary data on reverse-aging, which could be applied further for treatments of adult diseases.

617. Melamine Functionalized Copolymer for Sequence Specific siRNA Loading and Ligand-Driven Delivery

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Triamino triazine, also termed melamine, presents three identical interfaces that are capable of recognizing U/T with three hydrogen bonds. When displayed on a polymeric chain, melamine displaying polymers are capable of condensing structureless U/T tracts into triple-stranded structures, which have been conferred with enhanced tolerance towards nuclease degradation and preserved biological function. In this study, a mixed-block copolymer with alternating display of N-Acetylgalactosamine (GalNAc) and melamine $p(GM)_{10}$ is synthesized via reversible addition-fragmentation chain transfer (RAFT). The polymer has been demonstrated to sequence specifically load the 27-mer dicer substrate siRNA targeting ApoB-100 precursor mRNA that is appended with U stretches. The loaded siRNA can be efficiently processed by recombinant dicer, and readily uptaken by HepG2 cells via receptor mediated endocytosis, eliciting an IC 50 of 5 nM. Further, the copolymer is immunologically tolerant as the result of carbohydrate decoration, and induces minimum cytokine release syndrome. Hence, melamine displaying polymer offers a novel siRNA delivery strategy with enhanced RNAi potency; further, RAFT polymerization has been demonstrated to be a versatile platform for synthesizing tailored delivery system with tissue/disease-specific ligand conjugations.

618. Invivofectamine® Reagents for *In Vivo* mRNA Delivery for Research and Therapeutic Use

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mRNA has emerged as a promising new class of biologics since it lacks genomic integration and results in transient expression of the encoded protein. This makes it especially attractive for target ID validations, protein replacement, vaccines and gene editing for *in vivo* applications. However, mRNA is a large molecule and thus, difficult to stabilize and encapsulate for potent *in vivo* therapy. There is an urgent need to develop efficient delivery systems for organ specific *in vivo* mRNA delivery for research and therapeutic applications. At Thermo fisher, we have leveraged over 25 years of expertise in our delivery portfolio and developed novel lipid nanoparticles (LNPs) specifically for *in vivo* applications. Our approach involves implementing Design of Experiment (DOE) which provides us the flexibility to optimize formulations for organ specific delivery. The process of formulating these LNPs is simple, scalable and results in uniform-sized LNPs with a narrow PDI. The LNPs efficiently encapsulate and protect the mRNA from degradation and facilitate cellular uptake which translates into efficient delivery and reduced toxicity *in vivo* We have myriad LNPs that can efficiently deliver mRNA to the Liver, Lung, Spleen, tumor

(IT), vaccine (IM, ID, SC) and gene editing applications. For example, we have successfully shown *in vivo* delivery of Cas9 mRNA and LoxP guide RNA (gRNA) to induce expression of floxed tdTomato in the liver, Spleen and Lungs in reporter mice. We are also currently working to demonstrate gene editing of therapeutically relevant targets like FVII and PSCK-9 using modified gRNA and Cas9 mRNA. These LNPs complexed with a chemically modified, luciferase-encoding mRNA (Luc mRNA) exhibit luciferase activity in the order of 10^{11} p/sec *in vivo*. The liver LNP is 100X more potent than our previous generation Invivofectamine 3.0 (IVF 3.0) and has shown to significantly boost therapeutically relevant proteins like serum mouse erythropoietin (EPO) and human Factor IX (hFIX) levels in mice after a single injection. It is also 400% more effective in knocking down blood FVII levels in mice as compared to IVF 3.0 The Lung LNP delivers mRNA/siRNA exclusively to the lung tissue. When complexed with siRNA against an endothelial specific target, Tie2, over 90% knockdown was observed in the isolated lung tissue. Ongoing work with the Lung LNP involves knockdown analysis of additional cell type specific targets using siRNA to validate transfection of specific populations of cells within the lung tissue. We have also shown our LNPs can deliver mRNA into mouse hair follicles when applied topically on to the skin. These novel LNPs are also well tolerated and do not exhibit systemic toxicity. Our primary aim is to enable scientists by leveraging our delivery technology platform. We are striving towards this goal by launching commercially available Invivofectamine reagents that will reach a wider customer base and address their needs, thus accelerating the development of mRNA therapeutics.

619. Display of Single-Chain Variable Fragments on Extracellular Vesicles for Enhancing Recipient Cell Targeting and Uptake

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Extracellular vesicles (EVs) are nanoscale vesicles secreted by all cells that mediate intercellular communication through the transfer of protein and nucleic acids. These natural transport capabilities make EVs an attractive platform for the delivery of therapeutic biomolecules. In addition, the ability to engineer EVs and thereby customize the display of proteins on the EV surface to target specific recipient cells enhances the potential of these vesicles as therapeutic delivery vehicles. Most EV targeting investigations reported have involved displaying small peptides on the EV surface. While this approach confers modest enhancements in uptake for some peptide-target interactions, in general this strategy can be ineffective for reasons that are not yet clear. We hypothesize that targeting efficiency may be limited by the generally modest affinities conferred by peptide-target interactions (dissociation constants are typically in the micromolar range). Therefore, in order to increase the affinity of engineered EV-recipient cell interactions, we developed a method for displaying single-chain variable fragments (scFvs) on the surface of EVs. We evaluated the extent to which scFv display impacts EV uptake in a target-specific fashion, including the use of cells that exhibit low basal EV uptake. Our method could be used to promote high affinity recipient cell targeting as well as to direct EVs to particular uptake pathways with the goal of increasing functional delivery of therapeutic cargo.

620. Intrathecal Drug Delivery Platform for Gene Therapy: An *In Vitro* & *In Vivo* Delivery Study

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Intro: Optimization of gene modifying therapies for the treatment of CNS disorders requires precise, consistent and controlled delivery. Current methods and technologies used for precision therapeutic delivery often show poor biodistribution and lack of concentration at the desired anatomic target(s). A factor underlying these limitations is a lack of knowledge about cerebrospinal fluid (CSF) hydrodynamics and lack of engineered drug delivery systems to deliver therapies within the anatomically complex intrathecal space. The Pulsar™ intrathecal platform (Alcyone Lifesciences, Lowell, MA) utilizes CSF dynamics incorporated into a proprietary intelligent auto-injector pump system in conjunction with a unique lumbar puncture needle system or navigable multiport intrathecal catheter. This system is aimed to deliver highly disease and patient specific gene therapies by precision delivery. This abstract summarizes the preliminary *in vitro* and *in vivo* experiments performed to quantify the system's ability to distribute solutes in the intrathecal space. **Methods:** A state of the art optically clear 3D printed phantom of a healthy 23 year old female spine was designed and constructed (**Figure 1a**) based on high resolution MRI and computer aided design as described in our previously published work. A custom designed CSF flow pump was used to reproduce subject-specific CSF flow. Imaging was collected using a digital mirrorless camera. 5 mL of aqueous fluorescein sodium (66.4 mM) was injected over 1.5-5 minutes at L3/L4 with a 22-gauge spinal needle using the Pulsar™ platform protocol and compared to a manual bolus injection. Tracer spread was recorded by time lapse over 45 min. Digital image subtraction was applied to quantify spatial-temporal tracer distribution. *In vivo* preclinical testing was performed in Non-Human Primates (NHP) (7-9kg). 0.5-1 mL of 5 mM Gadolinium (Magnevist™) was delivered using the Pulsar™ platform utilizing proprietary 'pulsatile' flow rates and imaged using contrast-enhanced T1-FLAIR MRI. MR measurements were collected at time points corresponding to baseline (pre-injection) and immediately after delivery (5-15 minutes). MRI post-processing was performed using Amira image analysis software (FEI, Inc.). **Results:** In the *in vitro* human spine model, total solute transit time was ~45 min from L3/L4 to the cranial base. Precision delivery using the platform increased axial solute distribution in comparison to a manual bolus injection (**Figure 1a**). Transit time reduction to reach T8 was ~33 min. *In vivo* MR measurements showed rapid biodistribution of Gadolinium from L3/L4 to the cranial base immediately after the infusion, T_1 (**Figure 1b**). Several proprietary infusion profiles were demonstrated to either maximize Gadolinium in rostral regions towards the brain or widespread biodistribution throughout the CNS. **Conclusion:** These preliminary results support that the delivery system and infusion protocol can play an important

role in gene delivery axial solute distribution and should be considered alongside biologic interactions. The *in vitro* human spine model allowed characterization of solute spread within an anatomically realistic geometry. The *in vivo* NHP model allowed demonstration of solute spread based on various delivery profiles. These methods and findings assist in optimization of CNS drug delivery.

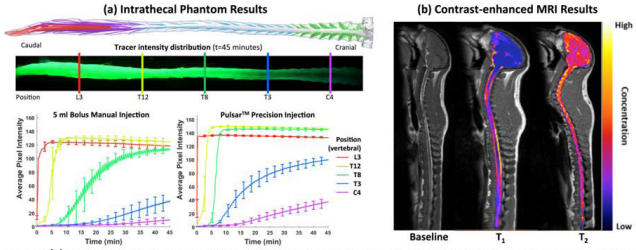

Figure 1 (a) Comparison of tracer distribution using the 3D printed intrathecal phantom (error bars represent standard deviation for three trials). (b) Contrast-enhanced MRI quantification of gadolinium biodistribution along the spine for a nonhuman primate.

621. Light-Controlled Gene and Drug Delivery by Azobenzene-Based Catanionic Vesicles

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The lack of targeted delivery and triggered release during traditional chemotherapy causes high toxicity in the human body and extends the treatment. The usage of nanocarriers for chemotherapeutic drugs aids to increase cancer treatment efficiency. Additionally, a potential therapeutic genetic material, siRNA, can be used to deactivate targeted mRNAs that cause inhibition of disease-associated protein production. The main advantage is using nanocarrier to design certain size nanoparticles that have the tendency of accumulation in tumor cells more than in normal cells. Thus, we have developed a novel method to deliver both siRNA and a chemotherapeutic drug to cancer cells via photo-assisted catanionic vesicles. Spontaneous catanionic self-assemblies are formed upon the interaction of an azobenzene based cationic surfactant and a conventional anionic surfactant. As a result of the photo-responsive property of azobenzene moieties, the photoresponsive mixture microstructure can be switched with light illumination from vesicles to either micelles, lamellar structures, or free surfactant monomers. Specifically, the unique photo-assisted transition of vesicles to free monomers will resolve two major obstacles in cancer treatment such as passive targeting of chemotherapeutics through cancerous cells and triggered release with light illumination. The photoresponsive catanionic vesicles are self-assembled nanocapsules that can be used for encapsulation and controlled release of substances by UV light exposure. In this work, the effect of hydrophobic tail of surfactants on particle size, charge and surface characteristics will be measured by small-angle neutron scattering, dynamic light scattering, zeta potential meter and cryo-transmission electron microscopy to create an ideal delivery vector. Furthermore, the encapsulation efficiency and transfection rates of Bcl-2 siRNA and paclitaxel co-delivery through MDA-MB-231 human breast cancer cells will be explored. Therefore, azobenzene based catanionic vesicles will be used as a co-delivery tool to develop siRNA based therapeutics with a hydrophobic anticancer drug. It is expected to improve tumor therapeutic efficacy by the usage of the novel photo-assisted catanionic delivery agent.

Vector and Cell Engineering, Production or Manufacturing II

622. CAR-T Cell Manufacturing with Clinimacs Prodigy

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The approval of CD19-targeted chimeric antigen receptor (CAR) T therapy for pediatric acute lymphoblastic leukemia and relapsed or refractory large B-cell lymphoma by FDA in 2017 marked a new era for cancer treatment. The integral component for the success of this promising therapy relies on reproducible CAR-T cell manufacturing. The manufacturing process for CAR-T cells is complex and includes T cell isolation, activation, transduction, expansion, formulation and cryopreservation. Ten years ago we established a robust modular CAR-T production platform that has allowed us to successfully manufacture more than 300 CAR-T cell products supporting 12 CAR-T cell clinical trials. In order to further increase the throughput in CAR T cell products at our center and to enable reproducible manufacturing at multiple point of care, we are now evaluating the CliniMACS Prodigy system, which incorporates all steps of the manufacturing process from T cell selection to end of process CAR T cell formulation. To date we have performed a number of runs to generate T cells expressing CARs encoded by gammaretroviral vectors. One run was initiated with a fresh apheresis derived from a healthy donor while 3 others were started with ALL, CLL and prostate cancer patient cryopreserved apheresis products, respectively. The first 3 runs were conducted with the TCT program (without spinoculation during transduction) and the last run was conducted with the TCT program (with spinoculation during transduction). The medium recoveries of CD3+, CD4+, and CD8+ cells during cell isolation were 50.7%, 61.7% and 50.2%, respectively. The medium total number of end of process cells for the four runs (ranging from 10 to 14 days) was 3.3E9. The medium transduction efficiency was 15.8% for runs using TCT program without spinoculation, and 63.2% for the run using TCT program with spinoculation. We also observed that T cells manufactured using the latter Prodigy TCT program display a high proportion of T cells with a central memory phenotype. We are currently evaluating the in vivo antitumor activities of these CAR-T cells in the NSG PC3 tumor mouse model. These encouraging preliminary results warrant further investigation of the spinoculation TCT program for CAR-T cell manufacturing using gammaretroviral vectors.

623. Design and Testing of Vector Producing HEK 293T Cells Bearing a Genomic Deletion of the SV40 T Antigen Coding Region

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Lentiviral vectors are typically produced using human embryonic kidney (HEK) 293T cells because these cells yield higher vector titers

compared to the parental HEK 293 cell line. HEK 293T cells were originally created by stably transfecting HEK 293 cells with a plasmid expressing the SV40 large and small T antigens. The SV40 large antigen is known to be capable of malignant transformation. Therefore, the presence of residual SV40 T antigen protein and/or coding sequences in viral vector stocks produced using the 293T cell line is a significant safety concern. We have used the CRISPR/Cas9 gene editing technology to remove the SV40 T antigen-encoding sequences from 293T cells transfected with a recombinant plasmid that simultaneously expresses Cas9 and two distinct sgRNAs corresponding to the beginning and the end of the T antigen coding region, respectively using tandem U6 promoters. Three out of the 176 cell clones screened by PCR revealed deletions of the T antigen coding sequence while 55 of the clones lacked the antigen sequence plus adjacent plasmid sequences. A Western blot analysis done with cell extracts prepared from the three T antigen null clones confirmed that the T antigen protein was not produced in these clones. Lentiviral vector titers produced using the three T antigen deletion clones were up to 1.5 x 10^7 TU/ml, while the titers observed with unmodified HEK 293T cells and HEK 293 cells were up to 4 x 10^7/ml and 1.6 x 10^6/ml, respectively. We are currently evaluating the capacity of the T antigen negative cells to produce high titer AAV vectors. These results indicate that the presence of the T antigen sequences in HEK 293T cells are not primarily responsible for high-titer lentiviral vector production. We anticipate that use of the T antigen deletion clones for manufacturing of clinical grade vectors may ultimately add to their safety.

624. The Use of Automation in the Development of Lentiviral Vector Producer Cell Lines

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The commercial advent of cell and gene therapy is driving the need for industrial scale production of therapeutic vectors to meet clinical demand. Scale up of lentiviral (LV) manufacture from adherent to suspension has partly addressed this problem but has also highlighted limitations with current transient transfection processes. One solution is to develop producer cell lines (PCL) enabling the production of large quantities of vector while reducing costs of input raw materials and potentially improving consistency between manufacturing runs. Oxford BioMedica (OXB) has been able to generate PCLs with titres equivalent or better than transient processes in both adherent and suspension processes. HIV-1-based LV PCLs were generated at OXB by stably transfecting a HEK293T.TetR cell line which constitutively expresses the Tet Repressor (TetR) protein, with inducible plasmids encoding HIV-1 Gag/Pol, HIV-1 Rev, VSV-G envelope and selectable antibiotic resistance makers. GFP genome was constitutively expressed by the PCLs. The generation of PCLs has been industrialised using a bespoke Automated Cell Screening System (ACSS) which uses state of the art automation. The ACSS enables the isolation of up to 2000 clones by automated cloning by limiting dilution in either 96- or 384-well format. Furthermore, the ACSS can perform routine passage and high-throughput (HTP) LV production/evaluation from clonal PCLs using various screening methods. The best LV PCL clones produced higher titre LV than obtained using the manual clone selection process, and also demonstrated the potential for higher titres than can be currently achieved using the current HEK293T transient transfection process. We have previously developed the TRiP system, which directs translational repression of the transgene during vector production using the bacterial protein TRAP. Depending on the transgene, this can result in significantly improved production titres (Maunder, et al., 2017). OXB is now developing HEK293T-based, serum-free suspension cell lines constitutively expressing the TRAP protein; clones have been obtained that are stable, allow several hundred fold repression of the transgene, and yield high LV titres. In conclusion, the isolation and screening of larger numbers of clones (>1000) using the ACSS leads to the selection of higher titre LV producing clones than previously achieved when screening smaller numbers of clones (100-200) manually. OXB now has capability of leveraging this technology in generating cell lines based on tet-repressor and/or TRiP system for serum-free, suspension production of viral vectors. *Maunder, et al., 2017. Nat Commun. 2017 Mar 27;8:14834. doi: 10.1038/ncomms14834.*

625. Enabling Tools for Virus Producer Cell Line Development and Engineering: Improved Manufacturing of Retroviral and Lentiviral Vectors for Gene Therapy

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Background The encouraging results obtained in clinical trials together with the approval of several viral vector gene therapeutic products support their growth over the next years. However the clinical-to-market transition increases pressure on the bioprocess an its improvement. Gammaretrovirus and lentivirus derived vectors are the chosen vectors for ex vivo applications such as, chimeric antigen receptor (CAR) T cells therapies. Their manufacture presents several challenges, one of them is the development of stable producer cell lines. Lentiviral vectors add the additional challenge of containing cytotoxic components. Infective particles titration methods are a bottleneck for process development being laborious and in some cases lengthy. Herein, we describe a novel strategy and novel enabling methods for establishing and smart screening high-titer virus producing clones. **Experimental approach** The establishment of stable cell lines for lentiviral and gammaretroviral vector production has traditional relyed on the successive transfection and screening for clones presenting the expression of the respective viral component expression cassette (e.g. gag-pol, envelope, etc.). Herein we introduced all the expression vector plasmids and only cloned the final producer cell population, screening clones for infectious vector yields. To efficiently establish the cell lines and screen for infectious vectors we developed several enabling molecular tools specific for viral vector production: i) a merged cloning-screening protocol, ii) single and dual targeted integration

systems, iii) label-free titration biosensors and iv) tight selection of gag-pol expression constructs. The cloning and screening were merged in a single step by making use of split-GFP, a green fluorescent protein separated into 2 fragments, S10 and S11, which fluoresce only upon transcomplementation. A cell population producing infectious virus with a S11 transgene is cloned and co-cultured with a target cell line harboring the S10 fragment. Viruses produced by the clone infect the target cells and reconstitute the GFP signal. Only the clones yielding high signal are isolated, avoiding storage and screening of low titer clones. This method was combined with targeted integration which confers flexibility to modify the producer cell by the use of recombinase mediated cassette exchange (RMCE) methodology. For lentiviral cell line development the lentiviral protease was modified reducing its cytotoxicity, and expressed using a tigh stable selection. **Results and discussion** The smart cloning-screening methodology enabled establishing gammaretrovirus producer cells generating up to $1x10^8$ infectious particles *per* mL. This method combines both the power of clonal cell resolution with the dynamics of cell population analysis. It was used to evaluate different, cell substrates, expression vectors and metabolic cell engineering strategies. For example, through the over-expression of metabolic genes improved clones producing ten-fold fold higher titers were obtained. The method was modified for lentiviral vectors, stable producer clones constitutively and continuously producing above $1x10^6$ IP ml^{-1} day^{-1}, over 10 consective days were obtained. The combination of the novel enabling molecular tools permited a faster gene therapy viral vector development by tackling different important aspects of viral vector production as, high-titer clone selection, cell engineering and detection of infective vectors.

626. In Vivo Comparison of the Biological Potency of rAAV9-Microdystrophin Made by Transient Transfection and a Scalable Herpesvirus System

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A major challenge for systemic adeno-associated virus (AAV) gene therapy is to scale up vector production. Unfortunately, the potency of the vectors generated by scalable manufacturing methods is rarely compared with the ones made by the traditional transient transfection (TT) method, the method most often used in research studies. Here we blindly compared the therapeutic efficacy of an AAV micro-dystrophin vector generated by either TT or the herpesvirus (HSV) manufacturing system in 7-week-old mice from the mdx4cv model of Duchenne muscular dystrophy (DMD). AAV was injected systemically at the dose of $5x10^{14}$ (high, n=3), $5x10^{13}$ (medium, n=6), and $5x10^{12}$ (low, n=3) vg/kg. Micro-dystrophin expression, muscle histology and function were evaluated 14 weeks later. Comparable levels of micro-dystrophin expression were observed by immunostaining and western blot at each dose in a dose-dependent manner irrespective of the manufacturing method. Evaluation of muscle degeneration/regeneration by centronucleation quantification showed equivalent protection by vectors made by either method in a dose-dependent manner. Specific muscle force and the eccentric contraction profile were similarly improved in a dose-dependent manner irrespective of the vector production method. Collectively, biological potency of the AAV micro-dystrophin vector made by the large-scale HSV method is comparable to that made by the TT method. Our results support the use of the HSV-based system for large-scale AAV production in future clinical trials.

627. Development of a Safe AAV Manufacture Platform Using Plasmid DNA and Next-Generation dbDNA Technology

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Production of AAV vectors for gene therapy applications presents many challenges. Each process step initiating at the DNA production, followed by cell build through to upstream and downstream processing and quality control of the viral vector product requires thorough development and understanding in order to generate high quality material to supply the growing demand for clinical trials. Poor understanding of the recombinant AAV genome packaging efficiency and the ability of AAV to package off-target DNA sequences such as antibiotic resistance, structural and helper genes required in the transient transfection process, present an important safety consideration for clinical manufacture of AAV vectors. Here we describe the development of AAV manufacture platform aimed at addressing some of these key challenges of the production processes through (1) developing a media that maintains cell health during AAV production process; (2) developing an analytical toolbox for mis-incorporated DNA sequence analysis; and (3) using alternative next-generation DNA technologies in order eliminate the possibility of packaging of antibiotic resistance genes. A nutrient supplement for commercially available chemically-defined GMP-compliant media which maintains the metabolic requirements for the cells in AAV production process was developed. As a result, enhanced growth kinetics of HEK293T grown in suspension media was observed. Transient transfection for AAV production using plasmid DNA encoding transgene, essential AAV replication and packaging components in the presence of supplements improved transgene packaging efficiency as determined by qPCR assays while the total yield of AAV detected by ELISA was maintained. Mis-incorporated sequence packaging such as Helper, Kanamycin resistance gene and RepCap was analysed using qPCR assays on AAV2 material produced and purified via our platform process. The third aspect of this work was the evaluation of DoggyBone DNA™ (dbDNA™) - a novel, patented DNA amplification technology - in AAV production. This technology is high-yielding, high-fidelity, eliminates antibiotic resistance and is capable of gram-scale amplification of unstable sequences (e.g. secondary structures such as inverted terminal repeats) using an inexpensive benchtop process. The relevant dbDNA constructs carrying adenovirus helper genes, AAV2 RepCap and transgene were synthesised and used for transfecting HEK293T cells in a small-scale

and a medium-scale platform AAV manufacture process. Plasmid DNA-derived and dbDNA-derived AAV yields were compared on genomic and total particle titres. No significant differences were observed in the total particle yield indicating that dbDNA can be successfully used for AAV manufacture. An increased proportion of genome-containing particles was consistently observed in AAV derived from dbDNA, indicating improved packaging efficiency of the payload (GFP) using these constructs. Packaging of Helper and RepCap sequences was found to be similar to those observed in plasmid DNA-derived AAV. In addition, this work highlights the complexities associated with accurate determination of this type of residual packaging and interpretation of the quality of the final AAV product.

628. Scalable Purification of Adenovirus Serotype 5 Viral Vector Using Ion-Exchange Mustang Q® Membranes

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Due to the growing interest in the use of viruses for gene therapy and vaccines many virus-based products are being developed. The manufacturing process for viruses poses new challenges both for process development and for regulatory authorities tasked with assuring quality, efficacy and safety of the final product. For each process, the design of a suitable purification strategy will depend on many variables including the vector production system and the nature of the virus. A case study will be presented here to illuminate some challenges and solutions associated with downstream purification process development. Adenovirus is used in this study as part of a therapy for Type 1 diabetes. Currently, diabetes affects more than 400 million people worldwide and causes 3 - 4 million deaths annually. To restore insulin production capability to patients with Type 1 diabetes, Orgenesis♦ is using a cell therapy approach. This approach manipulates a diabetic patient's own liver cells outside of the body (*ex vivo*) via viral transduction with Adenovirus serotype 5 (Ad5). Traditionally, this virus is purified using Cesium Chloride (CsCl) gradient centrifugation steps. Aside from being slow and cumbersome, the CsCl gradient centrifugation purification method is not scalable. To adapt purification of Adenovirus to a practical, manufacturing platform we have developed a purification process which uses membrane chromatography. For these studies, a total of 6 x 10^{11} Infectious Units (IFU) of excreted Ad5 was generated in each Toxicology (Tox) run in the iCELLis® Nano bioreactor. Following harvest, clarification and sterile filtration steps were performed immediately. Clarified harvest was then loaded into a Pall Mustang Q anion exchange (quaternary amine) membrane in bind/elute mode. A tangential flow filtration (TFF) step was performed using a Pall 100 k Molecular Weight Cutoff (MWCO) TFF membrane for final concentration and buffer exchange. The Mustang Q membrane purifies virus from clarified harvest in a single bind/elute chromatography step and provides the greatest clearance of Host Cell Protein (HCP) and Host Cell DNA (hcDNA) from Adenovirus. This purification step provided ≥78% step yields, >500x reduction of serum and host cell protein, >30x reduction of hcDNA and high ratio of full vs. empty capsid (>90%). For the production of toxicological material, three runs were performed with the iCELLis Nano bioreactor and purified with a Mustang Q device (60 mL bed volume). This device was loaded with clarified HEK293 cell supernatant, which is in Minimum Essential Media (MEM) with 8% serum (no lysis step) at pH 7.3, 12-18mS/cm. Figure 1: Purification of Ad5 by Mustang Q membrane, cleaning impurities and providing >90% full capsid Clearance of serum protein, host cell protein and host cell DNA was provided through the Mustang Q membrane step. Based on the results from the Adenovirus assay, recovery from the crude harvest through the drug substance was greater than 75% for all Tox animal runs. Purified Ad5 from Tox runs was used in a Tox study and this material demonstrated comparable results to the Ad5 material produced using a CsCl gradient centrifuged conventional non

629. Abstract Withdrawn

630. Autologous Cryopreserved Leukapheresis Cellular Material for Chimeric Antigen Receptor (CAR)-T Cell Manufacture

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Tisagenlecleucel is the first United States Food and Drug Administration-approved CD19-directed genetically modified autologous T cell immunotherapy indicated for treatment of patients up to 25 years of age with B-cell precursor acute lymphoblastic leukemia (B-ALL) that is refractory or in second or later relapse. Tisagenlecleucel is manufactured using cryopreserved autologous leukapheresed cells as a key starting material. Tisagenlecleucel product manufactured by this process in centralized facilities has been used successfully in two global clinical trials for treatment of relapsed/refractory pediatric B-ALL (ELIANA; NCT02435849) and adult diffuse large B-cell lymphoma (JULIET; NCT02445248). Here we describe the rationale and benefits for use of cryopreserved leukapheresis material. The manufacturing process for tisagenlecleucel starts with leukapheresis, whereby blood mononuclear cells are collected from the patient. To be eligible for manufacture, a minimum total nucleated cell count is required. The leukapheresis material is cryopreserved within 24 hours after collection and shipped to the manufacturing facility where it is thawed, enriched for T cells, activated *ex vivo*, and transduced with the lentiviral vector containing the anti-CD19 CAR transgene. Transduced T cells are subsequently expanded, washed, formulated, and cryopreserved. Following final product release testing, cryopreserved tisagenlecleucel cells are shipped to the infusion site, thawed, and infused into the patient. Given the logistic challenges associated with manufacturing autologous CAR-T cells in a global setting, cryopreservation of the leukapheresis material provides more flexibility compared to using fresh starting material, which can show signs of reduced viability as early as 24 hours post-collection. Published studies have shown minimal impact of cryopreservation and extended storage on subsequent T-cell proliferation or phenotype following thaw. The cryopreservation process used for tisagenlecleucel manufacturing has been evaluated extensively including the cryopreservation media, cryobags, storage, cryoshippers, and thawing conditions. Using the current manufacturing process, with the preferred cryopreservation cocktail and controlled rate

freezer, high success rates were achieved with regard to manufacturing batches that met specifications and were released to patients. Based on the currently established manufacturing process for tisagenlecleucel, key advantages of using cryopreserved starting materials are that they (1) provide flexibility for patients to undergo leukapheresis when timing for treatment is most appropriate (2) provide in-transit protection of cryopreserved leukapheresis materials, and (3) facilitate global shipment and receipt of material, enabling patient access and business continuity across a global manufacturing network. A reliable and consistent manufacturing process has been established for tisagenlecleucel using cryopreserved starting material, which results in no significant negative effects on either the starting material cell function or manufactured CAR-T cell product. The consistent product quality and positive clinical outcomes achieved using the robust, validated commercial manufacturing process for tisagenlecleucel support the use of cryopreserved leukapheresed cells as the starting material for CAR-T cell manufacturing.

631. Evaluation of a New Pan-AAV Affinity Resin for rAAV Purification and cGMP Manufacturing

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Affinity interaction based resins are the foundation for the platform purification of many different biological molecules. In the purification of rAAV based therapeutics, the affinity resin AVB Sepharose HP (AVB) has been used to successfully purify several different serotypes, both on the bench and for cGMP manufacturing. However, growing interest in capsids that do not bind to AVB, the desire for platform processes, and increasing scales of rAAV production have created the need for a true pan-AAV affinity resin with high capacity across all serotypes. In this work we investigate the use of a new affinity resin, POROS CaptureSelect AAVX (AAVX), for the purification of various serotypes from both crude and pure feed streams. Performance of AAVX is directly compared against other commercially available AAV affinity resins. Differences in capacities, binding kinetics, loading approaches, wash step tolerances, cleaning options, elution approaches, and other chromatographic critical process parameters, as well as the scalability and suitability of the new resin for commercial processes, is discussed. The results demonstrate that the new AAVX resin is a viable option for the scalable, platform purification of various rAAV capsids from crude feed streams.

632. Validation of a Fluorescence-Based Staining Method to Determine CAR T-Cell Products Viability

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Determination of cell viability is an essential test during the process of manufacturing CAR T cells, from in vitro expansion step to formulation and final release of CAR T cell products for clinical use. Fluorescence-based viability methods have greatly evolved in the past ten years offering more reproducible, non-subjective results and semi-automated platforms. Our first objective was to compare the Acridine Orange/Propidium Iodide (AOPI) method on the Cellometer platform (Nexcelom Bioscience) with our current method based on Trypan blue exclusion (TB) to provide the foundation of a method change. Our second objective was to eventually revise viability release cut-off according to the new method and implement its use in our analytical and manufacturing processes. During CAR T cell manufacturing, final products were assessed in parallel by TB and by the AO/PI in triplicate 1- before cryopreserving the product into infusion bags and 2- after thawing a sentinel aliquot to evaluate the viability after one cycle of freezing/thawing. A total of 50 CAR T cell products were tested before cryopreservation and 155 after thawing. 1928z CAR T cell products accounted for 74% of the products. Viability results were strongly correlated between the two methods ($r>0.78$) with AO/PI viability results consistently lower ($p<0.001$) than TB viability results. The average difference was greater for fresh products (-11.6%) than for thawed products (-5.7%). Interestingly the difference in viability between paired fresh and thawed products was consistent for the AO/PI method and inconsistent for the TB method suggesting that TB method grossly overestimates viability of fresh products. In conclusion the AO/PI is more sensitive and more reliable to assess the viability of fresh and cryopreserved CAR T cell products. In order to implement the new method, viability release criteria were revised and new cut-offs have been submitted to the FDA for IND amendments.

633. Development of Highly Automated TEM Assay for Adenoviral Vector Purity and Integrity Analysis

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Transmission electron microscopy (TEM) can be used to study virus-based gene therapy vector products and intermediates. However, the manual acquisition of images and the classification and counting of particles is a slow process and constitutes a significant bottleneck in analysis. For analysis of viral vector preparations it is beneficial to quantify the number and integrity of virus vectors in a sample against a background of non-viral material. We have been testing, optimizing and further developing semi-automated image analysis for negatively stained adenoviral vectors, using a MiniTEM-system. The system facilitates automated image acquisition, resulting in high number of micrographs. Our aim was to develop an assay which could be used for adenoviral drug products as well as processing intermediates. The assay was based on a script which recognizes vector and debris particles and classifies them into subcategories: Intact and empty/broken vectors and different types and sizes of debris. Vector purity and integrity were primary assay outputs, but other outputs were also tested and

developed, such as vector clustering. We started with an existing, non-optimized virus particle recognition script. The script was further developed and optimized, and new assay functions were added as new staining outcomes or research questions were encountered. High variation in negative staining complicated the assay development. Due to the time required to analyse several grids per sample, virus particle recognition was set to a compromise between script accuracy and staining quality requirements. Staining variability also greatly affected the threshold selection for debris recognition and increased total turnaround time. With automated image acquisition and analysis, the main factors affecting the assay hands-on time became the changing of grids and the manual waypoint search. Despite the compromises made, the assay performs its essential functions and its results have benefited the development of adenovirus vector manufacturing.

634. Producer Cell Line Engineering for Large Volume Manufacturing of Therapeutic AAV

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Recombinant adeno-associated viruses (rAAV) remain one of the most promising gene therapy vectors for patients with genetic abnormalities. rAAV can safely deliver long-lasting expression of a therapeutic transgene. To create a rAAV virus the wild-type viral genome is replaced with a functional transgene expression cassette. A constitutive promoter can be utilized to drive strong expression of the transgene once the rAAV vector has infected the target cell. Unlike wtAAV, the recombinant vector avoids the pitfalls of genomic integration by establishing extrachomsomal episomes or concatemers. Multiple studies have shown that rAAV can provide sustained expression of the transgene in cultured cells and pre-clinical models, providing evidence that rAAV could offer a cure for certain diseases. Bioengineering advancements have expanded the viral tropism beyond the constraints of naturally occurring AAV capsids, increasing the types of cells that can be thought of as targets. Taken together, rAAV therapies have attractive qualities to safely address the needs of patients where protein or small molecule therapies would fall short. One challenge with therapeutic rAAV is the ability to generate enough virus for clinical trials and commercial supply. This is particularly true with neuromuscular or hemophilia patients in which doses can exceed 1 x 10^{14} viral genomes per patient. Typical yields from a rAAV production are around 10^{4} viral genomes per cell, meaning batch cell numbers would need to exceed 10^{10} for a single dose. This amount of therapeutic virus will require a production platform that can reliably generate sufficient quantities of therapeutic rAAV to meet patient demand. Biogen has selected the producer cell line (PCL) platform to meet the large demand for therapeutic rAAV. Producer cell lines are generated by stably integrating the AAV viral genes along with the ITR flanked therapeutic gene of interest into an *in vitro* host cell line. rAAV production is then triggered by the addition of a 'helper virus' to provide functional genes for AAV replication. Traditional PCL platforms have used HeLa cells as the host and Adenovirus type-5 to deliver helper functions. We have used this platform as a basis for further development. Presented here will be our rationale for selecting the PCL platform, improvements made to the platform for ongoing clinical support, and our vision for the next generation PCL platform.

635. All-in-one Delivery of Gene-Editing System and Antigens into Primary Cells Using a MS2-Chimeric Viral RNA Delivery Tool Designed for Clinical Applications

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Gene editing and immunotherapy show great promises for a wide range of therapeutic areas such as genetic disorders, cancer immunotherapy and antiviral strategies. Nevertheless, both therapeutic strategies face a number of challenges especially when you have to simultaneously deliver multiple sequences of interest in order to manufacture the next generation of cell-based medicines. Such sequences are gene editing nucleases, guides, antigens, and immunomodulators (antagonists, co-stimulatory molecules, maturation markers, cytokines···). When used on primary and stem cells, genetic material transfer delivered in several steps triggers toxicity and causes phenotype modifications, unsuitable with clinical use. Drawing on this, delivery tools must be designed to develop one-time therapies in order to improve cell manufacturing and characterization. Furthermore, manufacturers have to scale-up cell-based medicine production while keeping the best cell quality and consistency including the preservation of cell phenotype and viability. The challenge is to manage the delivery of multiple RNAs at once without toxicity and control the expression duration of genes of interest, with a production process adapted for industrial scale and clinical use. LentiFlash being a revolutionary RNA carrier succeeds in dealing with DNA delivery issues since RNAs are directly delivered and transiently expressed into the cytoplasm. Furthermore, this new technology efficiently transfers multiple RNAs into cells and tissues without integration. It also manages to deliver multiple RNA species into each and every cell type like T cells and hematopoietic stem cells. We establish here that LentiFlash particles carrying the CRISPR-Cas9 technology grant an efficient disruption of targeted genes. More specifically, LentiFlash was successfully used to deliver CRISPR/Cas9 and knock-out the PD-1, TCRa or CXCR4 genes into human T lymphocytes. We will present data showing one-time therapies combining genome-editing and immunotherapy approaches. Moreover, LentiFlash and lentiviral vectors benefit the same production process. Consequently, LentiFlash-mediated transduction preserves the viability and original cell phenotype and uses the entire cell manufacturing platform validated in clinical settings. These properties offering an important safety consideration for clinical development and human applications are yielded by these properties.

636. A New Platelet Lysate Alternative to Serum for Ex Vivo Transduction and Expansion of Human T Cells

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Adoptive immunotherapy with *ex vivo*-modified T lymphocytes (T cells) has emerged as a promising therapeutic strategy to treat various cancer and autoimmune diseases. T cells engineered to express

Chimeric Antigen Receptors (CARs) have shown high rates of clinical response in patients with hematological malignancies and even early indications of clinical activity in solid tumors. The manufacture of CAR T cell therapies typically begins with autologous collection of mononuclear cells via leukapheresis, followed by enrichment of the T cell population, and finally genetic modification with viral vectors and *ex vivo* expansion. The use of fetal bovine serum (FBS) as a supplement for T cell culture carries a risk of pathogen transmission as well as xenoimmunization against bovine antigens. Human AB serum, yet another cell culture option for T cells, has supply limitations and therefore may not be sufficient to meet the expected demand for immunotherapies. Human platelet lysate (HPL) obtained from transfusable donor platelets is widely recognized as a valuable alternative to both FBS and human AB serum for production of clinical cellular therapies. This study aims to explore the feasibility of using a new pathogen reduced HPL (PR HPL) for the *ex vivo* modification and expansion of human primary T cells. PR HPL is produced with a method that has been demonstrated to reduce pathogens, including enveloped and non-enveloped viruses. Cryopreserved human CD3+ cells purified from peripheral blood mononuclear cells using negative immunomagnetic separation were used for this study. Cells were thawed in media containing different concentrations of either FBS, AB serum or PR HPL and activated with soluble tetrameric CD3/CD28 antibody complexes in the presence of interleukin-2. After four days of culture, cells were transduced with a lentiviral vector to deliver a green fluorescent protein expression cassette and transduction efficiency was assessed at day two and seven post transduction. In a second study, cells were expanded in media containing the three supplements for about two weeks and phenotype analysis using cell surface markers was performed at the end of the expansion period. Transduction efficiency, growth kinetics and phenotype of T cells expanded with PR HPL, FBS and human AB serum were overall comparable. This study demonstrates the feasibility to use human PL for the *ex vivo* modification and expansion of primary human lymphocytes.

637. Demonstrating Scalable T-Cell Expansion in Stirred-Tank Bioreactors Using the ambr15

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One major hurdle in regenerative medicine manufacturing is the development of a reliable, efficient, and scalable method of cell culture. In this study, the ambr15 bioreactor system was utilized to produce T-cell expansion data representative of large-scale stirred-tank bioreactors and comparing growth in a bioreactor to growth in static cultures. This was achieved by initially proving that impeller driven mass transfer does not affect total yield with no medium exchange. Further experimentation also identified 7.2 > pH > 7.1, a seeding density of $5.0x10^5$ cells/mL, and an initial period without mixing as key attributes of a successful T-cell bioreactor culture. Armed with these process variables, two perfusion mimic schemes where 50% of the media volume was exchanged every other day and 35% of the media was exchanged each day were tested against batch-fed cultures in T-flasks maintained at $1x10^6$ cells/mL. Culturing in the ambr15 bioreactor system resulted in approximately a 2.5-fold and 3-fold increase in total viable cell yield over the static cultures for the low and high perfusion rates respectively. Greater increases would be expected in a fully developed bioreactor system equipped to perform true perfusion. The compounded media exchanges resulted in >20% reduction in total yield due to cells removed during the perfusion mimic. Cell diameter, nutrient, and metabolite measurements taken throughout the study indicate T-cell growth arrested between day 9 and day 13 from cell activation not mass transfer limitations. This proof-of-concept study outlines a scaled-down T-cell culture method that representative of current full-scale manufacturing methods, and has the potential to outperform current technologies when combined with true perfusion and media exchange rates of 100% per day normally seen in manufacturing environments without changing cell identity.

638. Testing Approaches for Virus Vectors Used in Gene Therapy: Novel Methods and Regulatory Expectations

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Ensuring the biosafety and quality of virus vectors used in gene therapy is achieved through a multi-tiered approach that examines several factors to establish product safety and manufacturing consistency. The manufacture of viral vectors is complex and challenging with a number of key process goals that need to be maintained to achieve scalable processes and ensure reproducibility of product. The steady increase in the use of virus vectors to produce ground-breaking gene based therapies has intensified the need for novel approaches to both manufacturing and virus testing that improves upon well-established techniques and streamlined testing. Based on the innovative nature of many gene therapy processes, customization of test methods has been critical for success. The use of state of the art techniques to improve and expand existing testing methods that examine process product related impurities, identity and viral safety provides a level of quality assurance that addresses current regulatory expectations. We provide an analysis of the regulatory requirements for cell substrate testing and characterization of gene therapy virus vectors. Current testing methods are reviewed and testing challenges for viral vectors are discussed. This presentation will focus on innovative techniques, address critical quality attributes and address the limitations of small lot size, lack of terminal sterilization, limited availability of starting materials and continued supply of reference standards.

639. Development and Optimization of a Novel Transfection Formulation for High Titer Recombinant Lentivirus and Adeno-Associated Virus (AAV) Production

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The tremendous success in clinical trials and FDA approval of several gene therapies in 2017 has led to an increased interest in the optimization and scale-up of recombinant virus manufacturing processes to produce large quantities of high titer recombinant

lentivirus and AAV. To address the need, we screened our lipid and polymer libraries using a functional titer read-out to identify a novel transfection formulation that provides robust titers for lentivirus and AAV production in both adherent, serum-containing cultures, and serum-free suspension 293-derived cell types. Scale-up and reproducibility are key attributes in large-scale manufacturing. Since transfection complex formation is a critical step for transient transfection; several key parameters were assessed and optimized: buffer composition, incubation time, and the volume of the complex formation. Proof-of-principle scaling experiments were performed in suspension 293 cells, ranging from multi-well formats to large shake flasks. Reproducibility of the transfection process was also addressed through functional titer determination of multiple virus batches manufactured over an extended time period. Different transfection technologies have different compositions, transfection efficiencies, virus production capabilities and subsequent functional titers. Multiple transfection technologies were compared in head-to-head studies for recombinant lentivirus and AAV production in suspension 293 cell types using functional titers. Interestingly, transfection efficiency, as assessed by a GFP readout, was not always predictive of lentivirus or AAV yield. Virus genomes were also assessed to increase our understanding of the total population of virus that was produced using different transfection methods. Our data demonstrate that transient transfection is a robust and reliable tool that can be harnessed for large-scale manufacturing of both recombinant lentivirus and AAV.

640. Insect Media Evaluation for Cell Growth and rAAV Production in an Sf9-Baculovirus Manufacturing System

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Expanding clinical efforts utilizing rAAV vectors have required continuous improvements in rAAV manufacturing. Particularly, increased product supply demands have fueled efforts to maximize volumetric productivity of the cell culture process. One avenue to improve volumetric productivity is the optimization of cell culture media. In this work we compare the performance of a range of commercially available Sf9-specific cell culture media. Cells were maintained for approximately 100 population doublings to evaluate media robustness and ensure full adaptation to the assorted media. Multiple rounds of vector production were performed using various AAV serotypes to investigate the impact of the different media on the quantity and quality of vector produced. Furthermore, terminal batch growth cultures were conducted to investigate differences in growth and metabolic profiles. The importance of media selection and optimization in platform development is demonstrated in this work by marked volumetric and cellular productivity improvement for our process. These results highlight the significant impact that the cell culture media has on the production of rAAV in the Sf9-Baculovirus manufacturing system.

Presidential Symposium & Presentation of the Top Abstracts

641. Translating RNAi for Huntington's Disease: Intra-Putaminal Delivery of AAV2/1.Mihds1 for Comprehensive Dosing, Biodistribution, Silencing and Safety in a Non-Human Primate

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Huntington's disease (HD) is a neurodegenerative condition caused by a CAG repeat expansion within the *HTT* gene, which produces mutant Huntingtin protein (HTT) and causes gain-of-function toxicity. HD is characterized by cell loss in the basal ganglia and overlaying cortex, with particular damage to the putamen, an area of the brain responsible for initiating and refining motor movements. RNA interference (RNAi) is a naturally occurring process that mediates gene silencing and is currently being investigated as therapy for dominant diseases such as HD. Previously, we have demonstrated that adeno-associated viral (AAV) delivery of a microRNA (miRNA) targeting *HTT* mRNA (miHDS1) to HD transgenic mouse striatum improved neuropathological and motor phenotypes. The current study tested the dosing and biodistribution of AAV2/1.miHDS1 in rhesus macaques using ClearPoint® real time-MRI guided intracranial delivery. ClearPoint® is an FDA approved minimally invasive surgical platform to deliver therapies directly to the brain, allowing us to use the same device and method that will be implemented in the clinic and allowing us to refine our delivery protocol and dose to safely achieve comprehensive coverage of our target tissue before translating our therapy to patients. Animals received vehicle or one of three escalating doses of AAV2/1.miHDS1 administered to the putamen. Animals showed no significant changes in body weight in vehicle or AAV2/1.miHDS1 treated animals for 6 weeks post-surgery, nor the development of any aberrant neurological signs using a non-human primate specific neurological rating scale. Additionally, day and night homecage activity remained stable throughout the 6 weeks following surgery. To fully characterize the biodistibution of AAV2/1.miHDS1, 34 tissue punches were taken from each putamen hemisphere. *HTT* mRNA expression was analyzed by QPCR to achieve a biodistribution map. There was a dose-dependent decrease in overall rhesus *HTT* expression across the entire putamen ranging from 22-45% relative to vehicle injected controls. Strikingly, animals receiving the highest dose of AAV2/1.miHDS1 achieved significant silencing of *HTT* out to the most rostral and caudal aspects of the putamen. The middle and low doses had significant overall silencing of huntingtin although the efficacy was more restricted to the central aspect of the putamen. This data will help to translate the application of AAV2/1.miHDS1 to Huntington's patients in the safest and most efficient way possible.

642. In Utero Gene Correction Mediated by PNA-Nanoparticles

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Genetic mutations can be diagnosed early during pregnancy, yet many monogenic disorders continue to cause substantial neonatal and pediatric morbidity and mortality. Early intervention—through intrauterine gene editing—could correct the genetic defect, potentially allowing for normal organ development, disease improvement, or cure. In utero gene therapy thus far has focused on stem-cell transplantation and viral-mediated gene delivery, methodologies that do not allow for correction of a gene in its endogenous environment. Our recent work showed that site-specific gene editing to correct disease-causing mutations can be achieved efficiently and safely in postnatal animals via the intravenous or inhalational administration of polymeric, biodegradable nanoparticles loaded with peptide nucleic acids (PNAs) and single-stranded donor DNA. Here we demonstrate that nanoparticles can be safely delivered to developing fetal mice and that nanoparticles loaded with PNA/DNA can correct a disease-causing β-thalassemia mutation in utero that results in sustained postnatal alleviation of disease. We first investigate the biodistribution of biodegradable poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) encapsulating fluorescent dye in the developing mouse fetus at select gestational ages (E15.5 - E18.5). Intravenous delivery of NPs via the vitelline vein results in robust nanoparticle accumulation in the fetal liver at E15.5, during the period of rapid hematopoietic stem cell expansion. After in utero NP treatment, there were no deleterious effects detected on fetal survival or postnatal growth under any of the NP delivery conditions tested. Next, we determine if in utero treatment with PNA/DNA nanoparticles targeting the β-globin locus can be used to correct the underlying genetic mutation and improve the disease phenotype in a transgenic mouse model of β-thalassemia. A single NP injection on day E15.5 resulted in sustained alleviation of measurable signs of disease in the mice from birth into adulthood. Treated mice showed persistent amelioration of anemia, marked improvement of red blood cell morphologies, reduced reticulocyte counts, reversal of splenomegaly and associated extramedullary hematopoiesis. Functional disease improvement was accompanied by a clinically relevant level of gene editing (6% - 9%) as measured both in adult bone marrow and fetal bone marrow stem/progenitor cells, with no detected off-target mutations. In addition, in utero PNA-NP treated mice show a clear and significant survival advantage (100%) compared to untreated controls (69%) at 500 days after birth (p=0.02). Our findings demonstrate the feasibility, safety, and efficacy of PNA-mediated gene correction in a developing mammalian fetus and establish an approach that could be used in the treatment of numerous genetic disorders.

643. Engraftment and Phenotypic Correction of Hematopoietic Stem Cells in Non-Conditioned Fanconi Anemia Patients Treated with Ex Vivo Gene Therapy

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Fanconi anemia (FA) is a DNA repair syndrome characterized by bone marrow failure, congenital abnormalities and cancer predisposition. Aiming at restoring the hematopoietic function in FA patients, we have initiated a lentiviral-mediated gene therapy trial in patients with the FA-A subtype, which accounts for 60-70% of FA cases. Patients' hematopoietic stem cells (HSCs) were first mobilized with G-CSF and plerixafor and collected by 2-3 aphereses. In two patients, immunoselected fresh $CD34^+$ cells were immediately transduced for a short period of time (<36h) with the therapeutic PGK-FANCA. Wpre* lentiviral vector, while in other two patients $CD34^+$ cells were first cryopreserved until patients experienced the decline in peripheral blood cell counts defined in the clinical trial. Based on the *in vivo* proliferative advantage of gene corrected FA HSCs transplanted into NSG mice (see in Rio, Navarro *et al*. Blood 2017) FA patients were infused with gene corrected $CD34^+$ cells in the absence of any conditioning regimen. Despite the low number of $CD34^+$ cells typically present in the BM of FA patients, significant numbers of $CD34^+$ cells were mobilized in eight FA-A pediatric patients (mean number: 4.3 million $CD34^+$ cells/kg), although $CD34^+$ cell yields after purification were highly variable among them. Patients were infused with a range of 0.6 to 1.4 million $CD34^+$ cells/kg, transduced at efficacies of 0.17 to 0.53 copies/cell. Despite the absence of patients' conditioning, the engraftment of low numbers of transduced cells was confirmed in all patients following the first weeks post-infusion. Moreover, a marked *in vivo* expansion of gene-corrected cells was observed both in BM and PB in all treated patients. VCNs determined at 6 to 19 months post-infusion showed the presence of 1-37% PB cells containing the therapeutic provirus. Insertion site analysis has been conducted in PB cells from the patient with the highest engraftment. This

study showed an oligoclonal pattern of reconstitution, engraftment of multipotent HSCs, and no evidence of insertion-site mediated clonal expansion. Analyses of resistance of BM progenitor cells to mitomycin C and evaluation of chromosomal fragility of PB T cells to diepoxybutane showed an evident phenotypic correction in the two patients that were infused with the highest number of corrected hematopoietic progenitors, implying that these patients currently exhibit the characteristic phenotype of FA mosaic patients. Our studies demonstrate for the first time that lentiviral-mediated gene therapy in non-conditioned FA patients results in the progressive engraftment and phenotypic reversion of gene corrected blood and BM cells.

644. Transplantation and Persistence of CRISPR/Cas9 -Edited Hematopoietic Stem and Progenitors Cells for the Reactivation of Fetal Hemoglobin in Nonhuman Primates

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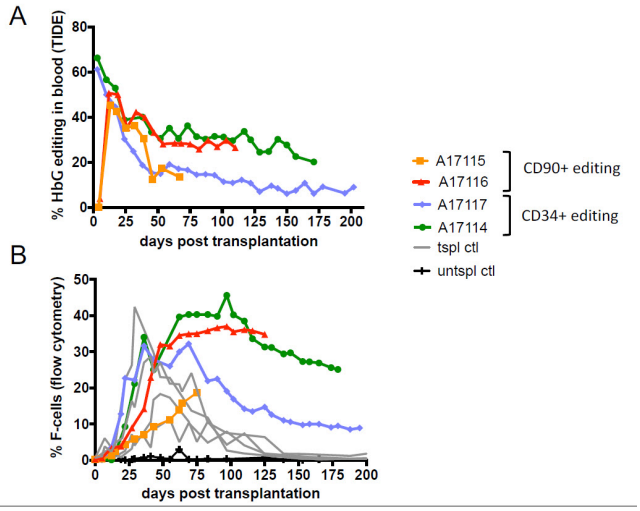
A promising therapeutic strategy for hemoglobinopathies consists in the genome engineering of patients' hematopoietic stem and progenitor cells (HSPCs) to reactivate fetal hemoglobin (HbF) production, which can serve as substitute for defective or absent adult hemoglobin molecules. Here, we used the nonhuman primate (NHP) large animal transplantation model toward clinical translation of this approach to ensure efficient gene editing in scale-up conditions and optimize long-term engraftment of edited cells. The CRISPR/Cas9 nuclease platform was employed to recapitulate a 13-nucleotide (nt) deletion in the gamma globin gene promoter identified in individuals with hereditary persistence of fetal hemoglobin (HPFH). Two rhesus macaques were transplanted with bone marrow-derived CD34+ cells edited *ex vivo* by CRISPR/Cas9 ribonucleoprotein electroporation. 70% editing efficiency was detected in the infusion product, with over 25% of cells containing the 13-nt deletion. Both animals showed rapid hematopoietic recovery and peripheral blood gene editing levels stabilized at 15% and 30%, respectively, at 6 months post transplantation (FigA). HbF production, as determined by peripheral blood F-cells staining (FigB) and quantitative PCR, was substantially increased in both animals as compared to controls and correlated with *in vivo* editing levels. To circumvent challenges associated with scale up and cost of editing reagents, we optimized our transplantation protocol by purifying a refined and more highly enriched target cell population ($CD34^+CD45RA^-CD90^+$), recently described by our group and capable of both rapid short-term and as durable multilineage hematopoietic reconstitution. Two rhesus macaques were co-infused with this CRISPR/Cas9-edited subset (comprising only 5-7% of total CD34+ cells) along with the remaining un-edited cells. *In vivo* gene-editing levels started at less than 5% but rapidly increased to 50% within a week, and persisted at efficiencies comparable to animals receiving edited CD34+ cells, consistent with this refined cell subset as major contributor to hematopoietic recovery (FigA). Taken together, these data demonstrate robust engraftment of CRISPR/Cas9-edited HSPCs following targeting of the 13nt-HPFH site in the NHP model leading to high levels of HbF production. In addition, we show efficient editing and engraftment of the CD90+ cell subset, an approach that reduces the required amount of editing reagents by 95%, circumvents challenges associated with scale up, without compromising editing or engraftment efficiencies, and thereby facilitating clinical translation of gene editing for the treatment of hemoglobinopathies.



A) Longitudinal analysis of gamma globin (HbG) editing in peripheral blood of transplanted animals as determined by TIDE analysis **B)** Frequency of circulating F-cells in transplanted animals as compared to control transplants (grey) and to an untransplanted control (black)

Innovations in CAR-T Therapies

645. A Simple Protein-Based Method for Generation of 'Off the Shelf' Allogeneic Chimeric Antigen Receptor T-Cells

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Introduction

Autologous chimeric antigen receptor (CAR) T-cells targeting CD19 have demonstrated revolutionary efficacy in the treatment of advanced B-cell malignancies. However, significant problems are associated with the use of autologous T-cells, including production time in patients with rapidly progressive disease, poor T-cell quality, production failures, as well as cost and logistics. Alternatively, an 'off the shelf' CAR-T product could be manufactured from allogeneic healthy donor T-cells, if these cells could be prevented from causing graft versus host disease (GvHD). GvHD is mediated by T-cell receptors (TCR) of donor T-cells. Previous approaches have therefore used genome-editing techniques to delete TCR expression, introducing

complexity and expense to the manufacturing process whilst imposing risk through unpredictable genotoxicity. We have developed an alternative strategy: TCR-knockdown through expression of an ScFv which recognises the TCR, fused to a C-terminal KDEL endoplasmic reticulum (ER)-retention domain. Co-expression of TCR-KDEL with CAR and the sort-suicide gene RQR8 in a single vector results in obligate linkage of CAR/RQR8 with TCR down-regulation. This permits the simple manufacture of highly pure CAR+TCR- cells, avoiding the potential genotoxicity of genome-editing.
Results
We cloned several anti-TCR/CD3 ScFvs with C-terminal KDEL motifs, and introduced these to TCR+ cell lines or primary T-cells by retroviral transduction. While clones OKT3 and BMA031 had no effect on TCR expression, cells transduced with UCHT1-KDEL demonstrated complete downregulation of surface TCR. We subsequently generated constructs in which UCHT1-KDEL, a 2nd generation anti-CD19 CAR (4-1BB-zeta endodomain) and RQR8, (a sort-suicide gene expressing CD20 and CD34 mimotopes) were co-expressed, separated by self-cleaving 2A peptides. We demonstrated obligate linkage of CAR expression and TCR downregulation in transduced T-cells. We then undertook positive selection for RQR8 marker-gene using CD34 magnetic beads. Following selection, purity of RQR8+TCR-CAR+ T-cells was >99%. We demonstrated that CAR19-KDEL and conventional CAR19 cells demonstrated equivalent specificity, cytokine release profiles and cytotoxicity in 48hr co-cultures with a panel of CD19+ and CD19-cell lines. In addition, in long-term co-culture assays, no differences in proliferative capacity, markers of differentiation or exhaustion were seen. However, while CAR19 cells proliferated in mixed lymphocyte reactions against foreign PBMCs, CAR19-KDEL cells did not demonstrate alloreactivity. We compared CAR19 and CAR19-KDEL cells *in vivo* using the NSG-NALM6 tumour xenograft model. Both CAR19 and CAR19-KDEL were able to substantially delay tumour progression relative to control CAR or mock-transduced T-cells in a low-dose (0.2M) model. Finally, we developed a xeno-GvHD model in which irradiated NSG mice were injected with a high dose (5M) of CAR19 or CAR19-KDEL cells. While 100% of mice in the CAR19 group died of xeno-GvHD by D65, all CAR19-KDEL recipients were alive and healthy at D180.
Conclusions
We have developed a novel, protein-based and non-genotoxic method for the rapid, simple and cost-effective generation of 'off the shelf' allogeneic CAR T-cells. This approach has the potential both to broaden access to CAR therapies and to reduce the financial burden of treatment.

646. Engineered Trans-Presentation of IL-15 Enhances Therapeutic Efficacy of GD2-Specific Car NKT Cells in a Xenogenic Model of Neuroblastoma

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Vα24-invariant natural killer T cells (NKTs) are an attractive effector for expression of tumor-specific chimeric antigen receptors (CARs) due to their natural anti-tumor properties and ability to preferentially localize to the tumor site in neuroblastoma (NB) and other solid tumors. We previously demonstrated that adoptively transferred human NKTs expressing GD2-specific CARs (CAR.GD2) more effectively localize to the tumor site than T cells and mediate anti-tumor activity in a xenogenic model of NB in NOD/SCID/IL-2Rγ^{null} (NSG) mice. We also found that NKTs can be inhibited within the tumor microenvironment (TME) and partially rescued by IL-15. These findings led to development of therapeutic NKTs co-expressing CAR.GD2 and IL-15, which are currently being tested in a phase 1 clinical trial (NCT03294954). Next, we hypothesized that the therapeutic potency of CAR.GD2/IL-15 NKTs can be further enhanced if IL-15 is engineered to be presented *in trans*, mimicking the physiological mode of IL-15 presentation in complex with IL-15Ra on adjacent cells. To that end, we transduced NKTs with retroviral constructs encoding CAR.GD2 for cell surface expression, and secreted forms of IL-15, IL-15Ra, an IL-15-IL-15Ra fusion, or IL-15 with IL-15Ra as separately secreted proteins. In another set of constructs, IL-15Ra or IL-15-IL-15Ra were linked to a single-chain variable fragment (scFv) targeting one of the following antigens, all highly expressed on NB cells: GD2, CD24, or CD56. We found that co-expression of cell surface CAR.GD2 and the described secreted molecules did not impact CAR expression in NKTs or their *in vitro* cytotoxicity against GD2-positive NB cells. However, NKTs co-expressing CAR.GD2 and fusions of the IL-15-IL-15Ra complex with the CD24 or CD56 scFv significantly increased long-term survival of NSG mice with metastatic NB xenografts compared with CAR.GD2/IL-15 NKTs. These findings reveal that dual targeting of tumor cells with a CAR specific for one antigen and an IL-15-IL-15Ra-scFv complex specific for another antigen overcomes the suppressive TME and activates NKTs for effective tumor elimination. This novel concept of immune engineering is broadly applicable to other therapeutic effector cells as well as other types of solid tumors.

647. Targeting CD19-Negative Relapsed B-Acute Lymphoblastic Leukemia Using Trivalent CAR T Cells

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B-Acute Lymphoblastic Leukemia (B-ALL) is the most common malignancy in children, and limited treatment options exist for patients with relapsed or refractory disease. Cellular immunotherapy, specifically chimeric antigen receptor (CAR) T cells targeting CD19, have demonstrated remarkable efficacy in treating B-ALL. However, recent reports show that up to 30% of patients who relapse after CD19 CAR T cell therapy have CD19-negative disease, justifying a need to expand CAR T cell therapy for B-ALL to include additional tumor-associated antigens. Here, we hypothesize that targeting three distinct leukemia antigens including CD19, CD20, and CD22 will improve B-ALL therapy outcomes and control disease progression during CD19-negative relapse. We designed a trivalent CAR T cell product with exodomains derived from single chain variable fragments (ScFv) targeting CD19 (FMC63 ScFv), CD20 (Rituximab ScFv), and CD22 (m971 ScFv). Our design incorporates viral 2A intervening sequences to achieve near equal expression of the three CARs individually on the surface of a single T cell (TriCAR). All CARs are fused to the intracellular signaling domains of the co-stimulatory molecule 4-1BB and the T-cell receptor zeta chain (2nd generation). Donor T cells were successfully engineered to express the CARs using a retroviral system, and the surface expression of these CAR molecules was confirmed by flow cytometry. Using a target expression validated panel of patient derived B-ALL cells (US7 CD19/CD20/CD22 +++/++/++, LAX-56 +++/+/+, TXL-2 +++/++/+++), we observed that TriCAR T cells killed ALL cells more robustly than CD19 CAR T cells at low effector to target (E:T) ratios. TriCAR T cells secreted similar levels of IFN-γ/TNF-α when compared to CD19 CAR T cells suggesting a safety profile similar to the CD19 CAR T cells, but with enhanced killing. In further analysis of single-cell killing dynamics via live cell tracking (TIMING) we found that TriCAR T cells exhibit more efficient killing of primary B-ALL as well as an increased frequency of serial killing compared to CD19 CAR T cells at low E:T ratios. We additionally interrogated immune synapse (IS) microcluster formation and actin dynamics during interactions between CAR T cells and B-ALL cells by ImageStream. TriCAR T cells formed significantly higher number of IS microclusters with increased actin polymerization compared to CD19 CAR T cells, suggesting distinct remodeling and enhanced cell activation when interacting with TXL-2 primary B-ALL cells. Further, we tested the efficacy of TriCAR T cells against primary CD19-negative relapsed bone marrow samples and CRISPR CD19 knockouts of the three primary ALL samples. Using these models of CD19 escape we showed that TriCAR T cells mitigated CD19 negative relapse, producing IFN-γ/TNF-α and killing CD19-negative primary ALL, while CD19 CAR T cells remained ineffective. Finally, we engrafted CD19 knockout Raji lymphoma cells (CD19-CD20++CD22++) into immune deficient mice. Further confirming our *in vitro* results, we found that TriCAR T cells effectively decrease tumor growth while CD19 CAR T cells are not efficacious. In conclusion, TriCAR T cells effectively target primary ALL cells with varying antigen profiles and mitigate CD19-negative relapse. This strategy has the potential for use as an initial CAR therapy in relapsed ALL or as a salvage therapy for patients with CD19-negative disease.

648. Immunosuppressive Population of Tumor-Associated Macrophages Expresses FRβ and Can Be Depleted with Specific CAR T-Cells to Control Ovarian Cancer Progression

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Tumor associated macrophages (TAMs) have been identified as key pro-tumor players in the microenvironment, and reported functions include improving tumor vascularization, matrix remodeling, and immunosuppression. Clinically, TAM density generally correlates with worse overall prognosis in most types of solid cancer. Folate receptor β (FRβ) is a myeloid-lineage antigen expressed on TAMs in human patients and in mouse models of cancer. Interestingly, the expression of FRβ is significantly upregulated on activated macrophages in inflammation sites, including the tumor microenvironment. Here, we tested the hypothesis that immunosuppressive properties are restricted to the FRβ-expressing subpopulation of macrophages and that those can be eliminated using FRβ-targeted chimeric antigen receptor (CAR) T-cells to disrupt the tumor microenvironment and control tumor progression. Using a mouse model of ovarian cancer (ID8), we corroborated that FRβ is expressed in a subpopulation of macrophages present in the ascitic fluid. We also found that this molecule is highly co-expressed with markers attributed to a M2-like phenotype of TAMs such as CD204, CD206, egr2, arg1, or IL10. On the contrary, FRβ negative TAMs expressed higher levels of M1-like markers, such as IL12 or IL1b. In order to test if, as suggested by the phenotypic characterization, FRβ-expressing and non-expressing macrophages have differential immunosuppressive capacity, we co-cultured T cells obtained from transgenic OT-1 mice (specific for $OVA_{257-264}$ peptide) with TAMs that had been previously sorted based on the expression of FRβ in the presence of specific peptides. FRβ-expressing but not FRβ negative TAMs were able to suppress T cell proliferation on a CFSE proliferation assay. In an ELISPOT assay, FRβ positive TAMs were capable of significantly decrease the amount of IFNγ-producing T-cells. Finally, we generated a CAR that targets mouse FRβ in order to deplete the FRβ-expressing population of macrophages *in vivo*. Administration of FRβ-specific CAR T-cells resulted in a comprehensive elimination of the FRβ positive TAM subset in the tumor microenvironment and produced a mild but statistically significant anti-tumor effect against FRβ-negative cancer cells. Future experiments will focus on the exploration of combinatorial approaches with conventional tumor-directed T-cell therapies. In conclusion, the targeting of FRβ-expressing immunosuppressive macrophages in the tumor microenvironment with CAR T-cells induces antitumor responses in solid tumors and

holds the potential to enhance the overall therapeutic efficacy of conventional CAR T-cell therapies that directly target solid tumor antigens.

649. Enhancing the Potency and Specificity of Tumor-Directed T Cells

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Several obstacles remain in translating the success of chimeric antigen receptor (CAR)-modified T cells against B cell malignancies to solid tumors. One common challenge, however, is the paucity of antigens exclusively expressed by the malignant cells, resulting in "on-target off-tumor toxicities" that can be potentially life threatening. Hence, to improve the tumor-selectivity of the transgenic cells, we generated tumor-specific molecular-pattern activated and regulated T-cells - "SmarT-cells" engineered to express three independent receptors designed to recognize a tumor pattern and containing customizable single endodomains that deliver either signal 1 (TCR activation), signal 2 (co-stimulation) or signal 3 (cytokine production). Therefore, unlike traditional CARs that are activated following engagement with a single antigen, SmarT-cells can undergo complete activation only in the presence of tumor-specific signature. To apply this strategy for the treatment of pancreatic cancer, we modified SmarT-cells to express a first generation CAR (signal 1), an inverted co-stimulatory receptor TBBR (TGFβ receptor II ectodomain fused with 41BB - signal 2) and a transgenic 4/7 inverted cytokine receptor (IL4 receptor exodomain fused with the IL7 receptor signaling domain - signal 3) that recognize prostrate stem cell antigen (PSCA), TGFβ and IL4, respectively. Indeed T cells modified to express CAR (97±1%, n=5) were able to lyse PSCA+ tumors (specific lysis - 41±5% - CAR vs 3±3% - TBBR vs 2±1% - 4/7ICR), while TBBR or 4/7 ICR modified T cells (67±14% and 87±5%, respectively, n=5) could harness their cognate cytokine (TGFβ or IL4) to enhance T cell survival and expansion, respectively (annexin-/7AAD- cells - 14±3% - CAR vs 32±7% - TBBR vs 12±8% - 4/7ICR, and fold change in cell numbers - 0.5±0.3 - CAR vs 0.5±0.3 - TBBR vs 9±3 - 4/7ICR). Furthermore, when co-expressed in SmarT-cells (61±5%-triple-positive cells, n=8), the customized receptors remained functional and provided additive benefits - tumor lysis (62±5% specific lysis, n=3), improved survival (29±1% annexin-/7AAD- cells, n=3) and enhanced proliferation (55±5 fold change, n=3) in presence of PSCA, TGFβ and IL4, respectively. Importantly, SmarT-cells selectively expanded only in the presence of the pancreatic tumor-specific signature (238±32, fold change, n=5), and remained inert in the presence of antigen alone (0.2±0.1, fold change, n=5). This preferential expansion was recapitulated in vivo resulting in selective anti-tumor responses (tumor volume 0 vs 68 mm^3, PSCA+TGFβ+IL4 vs PSCA alone by day 18), thereby demonstrating the safety and feasibility of our SmarT-cell approach for future clinical application. Furthermore, this strategy, which recapitulates physiologic T cell signaling has the additional benefit of rendering the transgenic cells resistant to otherwise suppressive molecules (TGFβ and IL4), and can be readily adapted to recognize specific tumor molecular signatures, by customizing the ICRs.

650. Optimization of CD28-Costimulated CARs for Increased Anti-Tumor Activity and Persistence

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Despite the stunning results of chimeric antigen receptor T cells (CAR-T) in patients with hematologic malignancies, the effectiveness of CAR-T cells in patients with solid tumors remains modest. A challenge to the field is a lack of significant expansion and persistence of the infused CAR-T cells in solid tumors. We have previously shown that the molecular design of CARs has a strong influence on T cell persistence. CAR-T cells containing the ICOS and/or 4-1BB intracellular domains (ICD) have shown improved persistence when compared to CAR-T cells containing the CD28 ICD in animal models of cancer. CD28 and ICOS are both members of the CD28 family and they both contain a YXXM motif that binds to PI3K in their intracellular tail. ICOS stimulation shows a stronger capability to activate PI3K than CD28, but CD28 can activate several other pathways, including Grb2. Here, we hypothesized that replacement of the YMNM CD28 motif for the YMFM ICOS motif within the CAR could improve the persistence of CD28-costimulated CAR-T cells. We show that signaling through the mutated CD28-based CAR (28z-YMFM) induced enhanced Akt phosphorylation with reduced PLCγ and Vav phosphorylation and reduced calcium flux when compared to conventional CD28-based CARs (28z). Despite these differences in T cell signaling, we could not observe any significant differences between the 28z and the 28z-YMFM CAR-T cells in terms of *in vitro* cytokine release or killing. We also investigated the therapeutic potential of the CD28 mutant when compared to CD28 and ICOS-based CARs (ICOSz). Treatment with 28z showed an initial decrease in tumor burden, but T cells did not persist and tumors eventually progressed. Animals treated with ICOSz showed a slower antitumor effect with enhanced persistence, that was not sufficient to induce complete responses. Interestingly, replacement of the CD28 motif YMNM for the ICOS YMFM motif enhanced T cell persistence at similar levels of those observed in animals treated with ICOSz CAR-T cells, which resulted in complete responses in all the animals treated. By contrast, incorporation of the YMNM motif into an ICOS-based CAR (ICOSz-YMNM) reduced the persistence and the antitumor effects of ICOS-based CAR-T cells. These results suggest that competition between Grb2 and PI3K for binding to the YMNM motif may be important in determining CD28-dependent downstream signaling events. All together, these results indicate that the poor persistence of conventional CD28-costimulated CAR T cells can be enhanced through mutation of the Grb-2 interacting residue, a genetic manipulation that can also increase the anti-tumor activity of the CAR-T cells.

651. Adoptive Cell Therapy with April Trimer Chimeric Antigen Receptor Shows Increased Anti-Tumor Efficiency against Multiple Myeloma

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Introduction: BCMA (B-cell maturation antigen) is an established target for chimeric antigen receptor (CAR) T-cell therapy of multiple myeloma. However, heterogeneous antigen expression and the risk of relapse with antigen negative disease narrow its therapeutic potency. Simultaneous targeting of two antigens by adoptive cell therapy may overcome these limitations. BCMA and TACI (transmembrane activator and CAML interactor) are members of the tumor necrosis factor (TNF) receptor superfamily and have both been shown to play an important role in promoting proliferation and survival of myeloma cells. Based on the structure of APRIL (A proliferation-inducing ligand), a natural ligand to both BCMA and TACI, we designed CARs that target the two likewise. We generated CARs to form APRIL monomers versus trimers and compared their anti-myeloma efficiency. Methods: We designed 2nd generation CARs targeting BCMA individually and BCMA and TACI concurrently: an anti(α-)BCMA CAR based on a single chain variable fragment, an APRIL CAR containing one truncated APRIL monomer and a TriPRIL CAR consisting of three truncated and fused APRIL monomers. All CAR constructs had CD8 as a transmembrane and 4-1BB-CD3ζ as an intracellular domain incorporated. CAR T cells (CARTs) were manufactured by lentiviral insertion of the CAR into primary human T cells that had been previously activated with CD3/CD28 coated beads. mCherry was used as a reporter gene to measure transduction efficiency. The capacity of the different CARTs to activate in response to and kill target cells was assessed by FACS analysis of the activation marker CD69 and luciferase based cytotoxicity assays. To determine the activity of our CARTs against BCMA and TACI both, in combination and separately, we used the multiple myeloma cell lines MM.1S and RPMI8226 (BCMA^{high} and TACI^{high}), as well as K562 cells transduced to express BCMA or TACI individually. Results: CARTs could be successfully manufactured for all three constructs, α-BCMA, APRIL and TriPRIL, with high transduction efficiencies (78-46%) from three different donors. When measuring activation in response to BCMA^{+} and BCMA^{+}TACI^{+} target cells, the α-BCMA and TriPRIL CARTs showed similar levels of activation (94-82% and 86-75%, respectively) while the activation of the APRIL CARTs was significantly weaker (58-45%; all values were normalized activation upon PMA/ionomycin stimulation). TACI-only expressing targets did not induce activation of α-BCMA CARTs (6%), induced moderate activation of APRIL CARTs (53%), and induced strong activation of TriPRIL CARTs (82%). Similarly, the α-BCMA and TriPRIL CARTs caused specific cytolysis of BCMA^{+}TACI^{+} target cells equally well at an effector to target cell ratio of 1:1 (96% and 93% respectively), while cell killing by APRIL CARTs was lower (83%). The APRIL and TriPRIL CARTs both killed target cells expressing TACI only (61% and 70% respectively), while no significant target cytotoxicity was observed by the α-BCMA CARTs (11%). Preliminary in vivo studies in xenograft models of myeloma demonstrate activity of these CARTs, and confirmatory in vivo studies are ongoing. Conclusions: APRIL-based CARs are an attractive mean to target multiple myeloma since they bind to both, BCMA and TACI, which are known to be expressed on nearly all malignant plasma cells. We were able to show improved multiple myeloma antigen recognition *in vitro* by a trimeric rather than monomeric form of APRIL fused to CAR signaling domains. Rational design of ligand-based CARs to promote multimerization may also prove beneficial when targeting other TNF receptor superfamily members.

Cardiovascular and Pulmonary Diseases

652. Gene Therapy Improves Lifespan and Cardiac Function in a Rat Model of Pompe Disease

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Introduction. Pompe disease is characterized by systemic depletion of acid-alpha glucosidase (GAA) resulting in ubiquitous lysosome glycogen accumulation. Cardiac dysfunction is prominent in early-onset patients with Pompe disease, yet the mechanism defining the development of cardiomyopathy is currently unclear. In order to elucidate the mechanisms which result in cardiac dysfunction, we have created a novel knockout model of Pompe disease that is more reflective of the disease pathogenesis observed clinically and serves as a critical model for evaluation of therapies. Methods. Zinc finger nucleases were designed to disrupt the rat Gaa gene, causing a ten base pair deletion that results in global knockout of GAA (KO). Male Sprague-Dawley rats were divided into the following groups: wild-type (WT), KO, and KO+AAV9-hGAA (KO+AAV). KO+AAV animals received a single intravenous administration of AAV9-hGAA at post-natal day 0. Animal body weight was recorded monthly and gross heart weight was collected at 5 months of age following euthanasia. Animals were subjected to in vivo physiological measures including cardiac MRI and electrocardiography (ECG). Biodistribution of transgene expression, toxicity, and efficacy following vector administration was assessed. Results. Deletion of 10bp in rat *Gaa* results in global knockout of Gaa enzyme activity, progressive lysosomal glycogen accumulation and early mortality. All males (9/9) that received AAV survived to 6 months, whereas KO rats had a median survival of 5 months and near 100% mortality rate at 6 months. Heart weight (WT 1.70±0.06; KO 2.12±0.09; KO+AAV 1.23±0.06 g) and mass ratio of heart to body weight (WT 0.29±0.01; KO 0.44±0.02; KO+AAV 0.25±0.01 %) was increased in KO animals when compared to WT and KO+AAV. ECG analysis of KO and KO+AAV animals show significant decrease in PR interval when compared to WT. KO animals display an elevation of R amplitude when compared to WT, while KO+AAV animals more closely reflected WT values (WT 0.34±0.04, KO 0.69±0.05, KO+AAV 0.41±0.05 mV). Cardiac MR revealed significant differences in KO and KO+AAV when compared to WT animals (WT 58.9±2.5, KO 64.2±3.9, KO+AAV 63.9±1.1 ejection fraction-EF (%); WT 483.1±31.7, KO

359.6±35.5, KO+AAV 547.5±29.5 end diastolic volume-EDV (ul); WT 196.8 13.3, KO 121.1±7.6, KO+AAV 199.0±15.8 end systolic volume-ESV (ul); WT 286.3±27.3, KO 238.5±33.4, KO+AAV 348.5±13.9 stroke volume-SV (ul)) 5 months post vector administration. Conclusion. Here we describe a novel rat knockout model of Pompe disease created by zinc finger nuclease (ZFN) technology, resulting in complete absence of the Gaa enzyme. Gene replacement via AAV administration results in widespread GAA expression. Key survival and cardiac measures show significant improvement in KO animals receiving AAV when compared to age matched KO rats. Our results give the opportunity to optimize and evaluate therapeutic potential of existing and next-generation therapies for Pompe disease.

653. AAV-Mediated *TAZ* Gene Replacement Restores Cardioskeletal Function and Improves Aberrant Proteomic Profiles in Barth Syndrome

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Introduction: Barth syndrome (BTHS) is a rare, frequently fatal, mitochondrial disease that affects heart and skeletal muscle and has no curative treatment. It is caused by recessive loss-of-function mutations in the X-linked gene *TAZ*, which encodes tafazzin. **Objective:** To develop a clinically relevant gene therapy to restore tafazzin function and treat BTHS, three different adeno-associated virus serotype 9 (AAV2/9) vectors were tested to identify the optimal promoter (cytomegalovirus, desmin, or tafazzin) for *TAZ* expression following intravenous administration of $1x10^{13}$ vector genomes/kilogram to a mouse model of BTHS as either neonates or adults. **Methods:** Biodistribution of *TAZ* expression, mouse activity assessments, fatigue in response to exercise, muscle strength, cardiac function, mitochondrial structure, mitochondrial respiration, and electron transport chain complex activity assays were evaluated to measure the extent of functional improvement in treated mice. **Results:** The desmin promoter (Des) provided strong *TAZ* expression levels to cardiac and skeletal muscle and resulted in significant functional improvement in all assessments. A quantitative tandem mass tag (TMT) protein labeling proteomics strategy was employed to compare expression profiles between healthy and BTHS mouse hearts and identified 111 proteins not previously recognized as being differentially expressed in BTHS including many known to play important roles in heart failure, cardiac development, biosynthesis pathways, and protein translation. The cause-effect relationships between tafazzin deficiency and altered protein profiles were confirmed by data demonstrating improved expression levels for 78.8% of the differentially expressed proteins in BTHS mice treated with dsAAV9-Des-*TAZ* either as neonates or as adults. **Conclusion:** This study provides substantial support for translation of an AAV9-mediated *TAZ* gene replacement strategy to the clinic using a Des promoter and identifies novel mechanisms of action for tafazzin relevant to BTHS as well as more common forms of heart disease.

654. Second Generation Gene Therapy for α1-Antitrypsin Deficiency Using a Genetically Modified α1-Antitrypsin Transgene Resistant to Oxidation

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Alpha 1-antitrypsin (AAT) deficiency is an autosomal recessive hereditary disorder associated with low serum levels of AAT, a serine antiprotease that serves to inhibit destructive neutrophil-released proteases including neutrophil elastase (NE). Due to an imbalance between protease and AAT activity in the lung, AAT deficiency is linked to early development of panacinar emphysema. While AAT normally provides highly effective protection of the lung against NE, AAT has an Achilles's heel -AAT can be inactivated by oxidants at active site methionine (Met) residues 351 and 358, rendering the AAT molecule incapable of inhibiting NE. Cigarette smoking accelerates the development of emphysema in AAT deficiency 20 year earlier due to oxidation of Met 351 and 358, rendering the AAT unable to protect the lung. Based on this background, we hypothesized that gene therapy with an oxidant-resistant AAT transgene would protect the lung in the context of an oxidant stress, providing a more effective therapy than with a wild-type AAT in the expression cassette. To evaluate this hypothesis, site directed mutagenesis was used to modify the Met351 and 358 residues to Val or Leu in the M1(Ala213) allele, a common normal AAT variant. Ten variants were assessed [residues 351, 358, M=Met, V=Val, L=Leu: MM (wild type), MV, ML, VM, LM, VV, LL, LV and VL] *in vitro* for the ability to inhibit NE alone or in the presence of oxidants N-chlorosuccinimide (NCS) or H_2O_2. Based on these studies, 4 AAT variants were packaged in AAV8 (AAV8 - MM, ML, VL and LL) and administered intravenously to male 6-8 wk old C57Bl/6 mice (n=5/group) at 10^{11} genome copies/mouse. After 4 wk, serum and lung epithelial lining fluid AAT levels were assessed by ELISA and NE inhibiting activity quantified in the absence and presence of oxidizing agents. All AAV8-hAAT administered mice expressed significant, persistent levels of human AAT in serum and epithelial lining fluid compared to vehicle (PBS) control mice. Importantly, variants with Val and/or Leu modifications to the 351 and 358 residues retained the ability to inhibit NE even in presence of high levels of oxidizing agents compared to controls. For example, AAV8-VL mouse serum samples retained 60% of NE inhibiting activity with the highest concentration of NCS tested compared to only 10% of NE inhibiting activity remaining with the same concentration of hAAT in AAV8-MM mouse serum ($p<0.0001$). Taken together, the data demonstrates that it is possible to establish systemic and lung levels of human AAT that could protect the lung from neutrophil elastase even in the presence of an oxidant stress. In the context that, even in nonsmokers or ex-smokers, the lung is exposed to oxidant stress from pollution and second hand smoke, this 2nd generation gene therapy for α1-antitrypsin deficiency should be more effective than the gene therapy with the wild-type α1-antitrypsin.

144. CAR-Tregs to Treat Heart Disease

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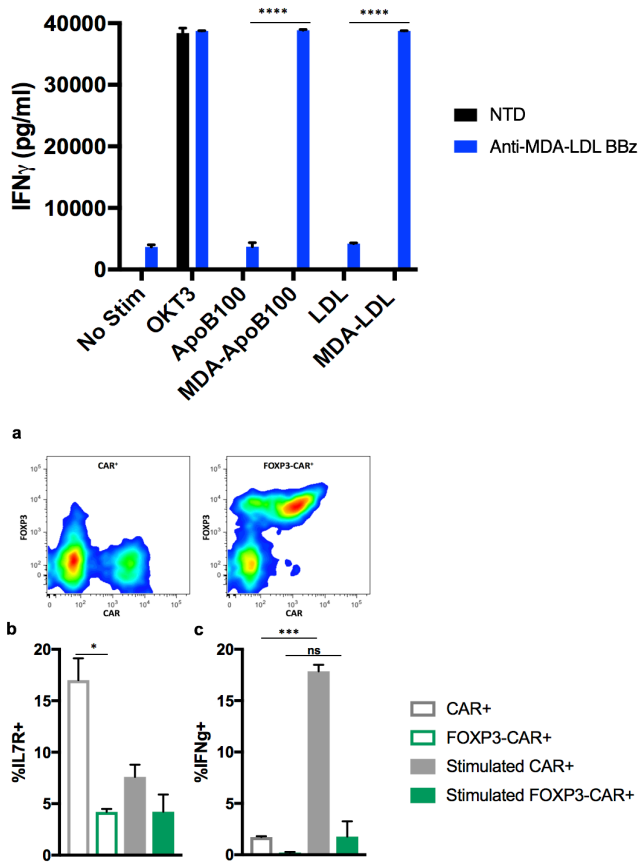
Cardiovascular disease is responsible for 17 million deaths worldwide annually, and cardiovascular disease from atherosclerosis accounts for 80% of these deaths. Atherosclerosis is a narrowing and hardening of arteries that leads to thrombotic events causing minimal oxygen perfusion to essential tissues. This plaque formation and rupture is thought to be due to progressive endothelial dysfunction from lipid deposition, resulting in inflammation and immune dysregulation. Recent evidence has implicated oxidative stress and the accumulation of modified low-density lipoproteins (LDL) with foam cell formation as part of the initiating factor of atherosclerosis. Currently, treatment options for atherosclerosis target LDL and not the subsequent inflammatory pathway that leads to plaque development. However, it has been found that patients with more anti-inflammatory cells, such as T regulatory (Treg) cells, experience less atherosclerotic events. Given the success of immune regulation and targeting with chimeric antigen receptor (CAR) T cell therapy, we hypothesized that this technology could be utilized for the treatment of atherosclerosis. The utilization of CAR-Treg cell therapies for inflammatory diseases, such as autoimmune colitis and multiple sclerosis, has previously been effective in murine models; therefore, we sought to develop a similar approach for the immune dysregulation in atherosclerosis by designing a CAR targeting malondialdehyde (MDA)-modified LDL. First, we incorporated the single chain variable fragment of a previously characterized MDA-LDL antibody into a lentiviral CAR backbone with a 4-1BB-CD3ζ intracellular signaling domain and confirmed expression on the surface of normal donor human T cells. We demonstrate that anti-MDA-LDL CAR is stimulated by low density lipoproteins and specifically reacts to MDA-modified LDL and not native LDL (**Figure 1**). We also developed CAR-Treg cells by transducing naïve human CD4 T cells with a bicistronic CAR vector that expresses FOXP3. After transduction and expansion, we confirmed that FOXP3 expression was significantly higher in our CD4 T cells transduced with FOXP3 (**Figure 2a**). Similarly, FOXP3-CAR$^+$ T cells exhibited low amounts of IL7R (CD127) post-expansion **(Figure 2b)**, consistent with the phenotype of naturally-occurring Treg cells. Lastly, upon stimulation through the endogenous TCR, FOXP3-CAR$^+$ T cells did not have any significant increase in IFN-γ production (**Figure 2c**), demonstrating a suppressive effect of FOXP3 expression. These initial studies demonstrate the feasibility of developing CAR-Tregs that could reduce the inflammation associated with modified LDL, potentially reducing macrophage foam cell formation and plaque progression.



656. Protease-Activatable Adeno-Associated Virus Vectors for Cardiac Disease Applications

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Heart disease is the leading cause of death in the United States, most predominately as a result of heart failure (HF). The current treatment methods work to reduce blood pressure, addressing the symptoms of HF. Despite these treatment options, the 5-year survival rate for HF is 50%, highlighting a need for more effective therapies. Gene therapy has been identified as a potential avenue to treat a number of cardiac diseases, and genes have been identified that have the potential to treat HF post myocardial infarction (MI). While these genes have shown promise, serious negative side effects can result from off-target delivery. In an effort to mitigate the risk of off-target delivery, we have developed a protease-activatable vector (provector) platform based on adeno-associated virus (AAV), which can be injected systemically and deliver genes more site-specifically to disease cells. AAV9 was chosen as the base vector for cardiac disease applications, as it is the most efficient AAV serotype for delivering genes to cardiac cells *in vivo*. The provectors contain a peptide insertion in the virus capsid which blocks cell binding. This "lock" blocks transduction at the cell binding step and can be removed by exposure to proteases,

which cleave specific sequences on each side of the "lock", releasing the lock from the surface of the virus capsid. This release of the "lock" allows the virus to regain its ability to bind cell surface receptors and transduce the cells. Following HF there is an upregulation of matrix metalloproteinases (MMPs) and cysteine-aspartic proteases (caspases) which provides local stimuli at the site of disease for the activation of the provectors. Provectors that are responsive to MMPs or caspases have been characterized *in vitro*, showing 100-fold ablation in transduction ability in the "locked" state. The provectors become "unlocked" after treatment with proteases, cleaving the sequences flanking the "lock". The activatability of these provectors ranges from 2.5 to 17x differences between the "locked" vs. "unlocked" states. Cell binding assays have confirmed that this difference in transduction is caused by the peptide insertion's ability to block binding of AAV9 to cell surface receptors when intact. The provectors have been characterized *in vivo* in a murine HF post-MI model. Compared to wild-type AAV9, the provector is able to deliver a reporter transgene site-specifically to the injury region of the heart, with decreased delivery to off-target organs. We are currently investigating the therapeutic efficacy of the provector delivering a clinically relevant transgene in the murine HF model.

657. *In Vivo* Genome Editing of PCSK9 in Macaque Liver Leads to a Stable Reduction in Serum Cholesterol

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Treatment of human genetic diseases by *in vivo* genome editing has tremendous potential, although successful translation of *in vivo* genome editing into the clinic will likely be hampered by many of the same issues that plagued gene therapy, such as insufficient delivery, immune toxicity, and insertional mutagenesis. There is a remarkable paucity of *in vivo* genome editing data in animal models, most of which have been restricted to mice. We have attempted to leverage our experience in AAV gene therapy toward the development of *in vivo* genome editing. In doing so, we focused on liver as a target using AAV8 vectors that have been successfully deployed in gene therapy clinical studies. PCSK9 was selected as a target, since its inhibition has been validated for treating cardiovascular disease via a reduction of serum cholesterol. Stable inactivation of PCSK9 could be accomplished through non-homologous end joining following a nuclease-mediated double stranded break. In this study, we utilized AAV8 vectors to evaluate the potential of engineered meganucleases to inactivate liver expression of the secreted protein, PCSK9, in non-human primates as a potential treatment for hypercholesterolemia. A single infusion of vector expressing the meganuclease resulted in a dose-dependent inactivation of the PCSK9 gene based on molecular analysis of liver DNA as well as a stable reduction of circulating PCSK9 and serum cholesterol for almost a year (still on-going). Analysis of liver tissue showed high frequency of on target indels that corresponded to the reduction in serum PCSK9. Animals experienced transient and asymptomatic elevations of serum transaminases due to the formation of T cells to the transgene product. Analyses of sequential liver biopsy samples obtained at day 17/18 and day 128/129 showed significant reduction of vector DNA, and transgene expression which declined to almost undetectable levels at the time of the second biopsy without any decline in the abundance of cells with edited PCSK9 genes. Molecular studies on liver biopsy samples demonstrated off-target cleavages that were reduced through the creation of a second generation PCSK9-specific meganuclease. These studies demonstrate very efficient and physiologically relevant *in vivo* genome editing in nonhuman primates and illuminate safety issues to be considered in clinical translation.

658. Development and Optimization of a PCSK9-Specific Meganuclease That Mediates Long-Term LDL Reduction in Non-Human Primates

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Individuals with Familial Hypercholesterolemia (FH) have abnormally high LDL levels that frequently result in early-onset cardiovascular disease. Proprotein convertase subtilisin/kexin type 9 (PCSK9) is an enzyme secreted primarily by hepatocytes that mediates recycling of the LDL Receptor. PCSK9 inhibition has been established as an effective treatment for many FH patients, making it an attractive target for in vivo genome editing. To this end, we have produced an engineered meganuclease that recognizes and cuts a target site in Exon 7 of the human/primate PCSK9 gene. The meganuclease mediates long-term reductions in serum PCSK9 and LDL levels following intravenous delivery to non-human primates (NHPs) with an AAV8 vector. Based on a thorough evaluation of the in vivo editing profile of the first-generation nuclease, we developed an optimized variant with similar on-target editing efficiency but greatly reduced off-target cutting. The improved specificity of the second-generation nuclease was demonstrated initially in mammalian cell culture and was then confirmed in a long-term in vivo study in NHPs.

Capsid Engineering

659. Characterization of a Protease-Activatable Adeno-Associated Virus Vector for Disease-Targeted Gene Delivery

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The broad tropism of adeno-associated virus (AAV) vectors is advantageous in some applications, but off-target effects can be detrimental in others. To address the issue of non-specific targeting, our lab has developed protease-activatable viruses based on AAV, called

provectors, for targeting the extracellular environment of pathologic tissue enriched with matrix metalloproteinases (MMPs), such as tumor microenvironments. These provectors will only bind and enter cells upon proteolysis of an inserted peptide 'lock' located in the virus capsid cell-receptor binding domain. Briefly, the peptide lock is a negatively charged motif that inhibits the virus from binding the cellular primary receptors and is flanked by MMP cleavage sequences. Upon proteolysis by MMPs, the negatively charged motif is removed and cell-binding functionality is restored, rescuing infectivity. Here we discuss the characterization of these activatable vectors both *in vitro* and *in vivo*. Firstly, we aimed to observe the provector's behavior in the presence of MMPs. The vectors were incubated with MMP-2 or MMP-9 and subsequently run on silver stain gels to observe cleavage profiles. We observed distinct shifting of the viral protein bands and a c-terminal fragment indicative of complete cleavage. Following these results, wild-type (WT) capsid vector and provector were tested for transduction efficiency before and after protease cleavage. Analysis by flow cytometry confirmed the provector is unlocked after MMP cleavage with 9.3x and 12.6x higher transduction of HEK293T and ovarian cancer HEYA8 cells, respectively, over 'locked' controls ($p<0.05$ and $p<0.001$). We next characterized the behavior of the provector *in vivo* to observe biodistribution, blood clearance, and immune profile. Vectors were injected intravenously into healthy mice, and viral genomes (VGs) were extracted from blood as well as several key organs and quantified by qPCR. Of note, provectors show significantly fewer VGs in the liver than WT (72x decrease, $p<0.05$), an important property if delivering cytotoxic payloads. We next studied the blood clearance of the virus. Interestingly the provector is cleared much slower than WT, suggesting that the ablated receptor binding by the inserted peptide locks mitigates vector removal from circulation (WT: 0.2% initial dose remaining at 4h, provector: 21% initial dose remaining at 4h, $p<0.001$). Finally, we aimed to characterize the cross-reactivity of raised antibodies by incubating WT virus with serum from mice injected with provectors. Of note, provector antibodies do not recognize WT capsids as well as WT antibodies do, suggesting that the provector is antigenically distinct from WT (provector Nab: 1:52, WT Nab: 1:3840). Continued *in vivo* studies are measuring the therapeutic effect of the provector platform in an ovarian-cancer mouse model. Together, these new findings show the promise of protease-responsive AAV vectors for targeted delivery to sites of disease.

660. Engineering Cell Type Specific Delivery Vectors for Noninvasive Modulation of Brain Circuits and Behaviors

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Within neuroscience the modulation and monitoring of specific circuits is achieved using a combination of transgenic animals and direct injection of viral vectors. To provide an alternative to invasive brain injection and increase transduction coverage and efficiency, we previously engineered adeno-associated viruses (AAVs) that efficiently cross the BBB in adult mice (Deverman et al, 2016; Chan et al, 2017). When delivering genes systemically, however, it is currently not possible to achieve a high degree of specificity for defined neuronal cell types in specific brain regions without the use of transgenic approaches. Here, we attempt to address this limitation by selectively engineering capsids with regional and cell-type specificity using CREATE, the Cre recombination-dependent AAV targeted evolution (Deverman et al, 2016) methodology that recovers capsids that transduce predefined Cre^+ target cell populations. Using a refined CREATE method and simultaneous *in vivo* selections in multiple Cre lines, we applied both positive and negative selective pressure by recovering AAV variants that transduce target neuronal subpopulations (e.g. dopaminergic cells) and off-target populations within multiple brain regions, and computationally excluding those variants that transduce off-target populations. After multiple rounds of selection, we have recovered variants that are enriched in target cell populations which, when coupled with gene regulatory elements, may enable efficient and specific transduction of defined cell populations without the use of transgenic animals. Another challenge faced with systemically delivered vectors is transducing target circuits and cell populations at sufficient copy number to drive neuronal activity. To this end, we provide proof-of-concept, noninvasive modulation of locomotion and reward in transgenic animals following systemic AAV delivery of chemogenetic actuators to dopaminergic circuits, a key target for multiple neurological disorders including Parkinson's. We coupled our systemic AAVs with Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), which are activated by otherwise inert compounds. We use the DREADD actuators to modulate locomotion and reward behavior after intravenous AAV delivery in transgenic TH-Cre mice. Movement of animals injected with AAVs packaging Cre-dependent excitatory or inhibitory DREADDs significantly increased (3-5 fold) or decreased (2-4 fold), respectively, after administration of the DREADD agonists CNO or compound 21. Additionally, animals conditioned to a specific chamber switched preference after systemic excitation of dopaminergic cells with CNO while placed in their non-preferred chamber, demonstrating a robust reward response following DREADD activation of TH^+ cells. Finally, we demonstrate tolerability of our systemically delivered AAVs when packaging fluorescent sensors of neuronal activity, such as GCaMP6, for *in vivo* 2-photon imaging. Together with chemogenetics and alternative methods under development, the AAV engineering approaches and vectors we are developing should provide researchers with minimally invasive access to defined cell populations, allowing for modulation and monitoring of distinct circuits and testing of behavioral effects in animals.

661. Safety and Increased Transduction Efficiency in the Adult Nonhuman Primate Central Nervous System with Intravenous Delivery of Two Novel Adeno-Associated Virus Capsids

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Adeno-associated viral (AAV) vectors represent an important new class of drugs for the treatment of diseases of the adult central nervous system (CNS). One of the major challenges of AAV gene therapy is delivering the transgene of interest to target cells at levels that are both safe and effective. Intravenous (IV) administration of AAV had provided relatively limited gene transfer to the adult CNS until the discovery of engineered capsids such as AAV-PHP.B (Deverman et al., 2016) and AAV-PHP.eB (Chan et al., 2017) that increase gene transfer to the adult mouse CNS by more than 40-fold compared with the previous standard, AAV9. A key translational step is to demonstrate in adult large mammals that AAV capsids substantially improve gene transfer to the CNS following systemic administration. We report here that two novel AAV capsids result in significantly enhanced transgene expression in the CNS of adult cynomolgus monkeys compared with AAV9. In our studies, an HA-tagged frataxin transgene was used to facilitate evaluation of biodistribution by immunohistochemical labeling for the HA tag. Significant transgene expression in the adult cynomolgus monkey CNS was observed at 4 weeks after treatment with the two novel AAV capsids but not AAV9, including regions such as spinal cord, brain stem, cerebellum, thalamus and substantia nigra. Numerous neurons were transduced in these regions, including those of therapeutic relevance to diseases such as ALS, Parkinson's disease and Friedreich's ataxia. Importantly, IV dosing of these novel AAV vectors in adult non-human primates was well-tolerated based on data to-date, including clinical signs, body weight, clinical chemistry and histopathology. These biodistribution and safety results in the adult non-human primate support IV dosing with these two novel capsids as a potential approach for the treatment of CNS disorders.

662. Engineering an All-In-One Light Activatable Adeno-Associated Virus Vector for Tunable Gene Delivery

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Attaining precise control over gene expression levels in targeted cell populations residing in dynamic microenvironments remains an intriguing, non-trivial challenge for contemporary clinical applications. We previously developed a light activatable adeno-associated virus (AAV) vector that leveraged the red/far red light responsive proteins Phytochrome B and Phytochrome Interacting Factor to control gene delivery [1]. Although promising, the platform requires multiple interacting components, which complicates translation beyond *in vitro* endeavors. Building upon insights from the aforementioned system, we aimed to create a single-component light activatable AAV platform in order to provide an improved and facile approach for fine-tuning gene expression in mammalian cells. We proceeded with the blue light responsive light-oxygen-voltage (LOV) domain because of its small size, well-characterized mechanism of action [2], and reliance on flavin chromophores found naturally in mammalian cells [3]. First, we redesigned an *Avena sativa*-derived LOV2 domain-based light-inducible nuclear localization sequence (NLS) [4] to satisfy structural and size constraints for insertion into the VP1 subunit of AAV. Additionally, we ablated the BR3 region in VP1 via site directed mutagenesis to render the viral NLS defective, for purposes of delineating the contribution of light activation to nuclear entry and cellular transduction. Wild-type (wt) and light activatable AAV expressing GFP were subsequently generated. Quantitative PCR was used to determine virus titers, and viral transduction efficiency was assayed by flow cytometry. Light induction experiments employed a custom light-emitting diode plate apparatus (LPA) [5]. We observed that high blue light intensities and exposure durations could markedly increase the transduction of wt AAV capsid vectors, likely attributed to significant temperature increases in the LPA. To eliminate this confounding factor, we optimized the bounds of light fluxes and illumination times used in the experiment, demonstrating in HeLa cells that heat-mediated transduction increase of wt capsid vector can be minimized. We are presently characterizing the dynamic range of our light activatable AAV system, namely the tunability of GFP expression in HeLa cells to varying blue light intensities. In summary, the various advantageous facets of our engineered AAV platform include design simplicity, minimal invasiveness compared to UV light/chemical inducer-controlled systems, and the potential to achieve unrivaled spatiotemporal resolution. References: [1] Gomez et al. ACS Nano. 10(1): 225-37, 2016. [2] Harper et al. Science. 301(5639): 1541-44, 2003. [3] Salomon et al. Biochemistry. 39(31): 9401-10, 2000. [4] Niopek et al. Nature Communications. 5: 4404, 2014. [5] Gerhardt et al. Scientific Reports. 6: 35363, 2016.

663. Characterization of a Novel Serine/Threonine Motif in the N-Terminal Region of Adeno-Associated Virus

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There are ~7,000 monogenic disorders affecting millions globally to which there are no curative treatments. Adeno-associated viruses (AAVs) are some of the most extensively researched and clinically tested viruses for gene therapy. Despite pre-clinical success, many cellular and

tissue barriers impede adequate AAV gene delivery and expression in clinical application. The AAV capsid has operative roles in cell binding, entry, endosomal escape, cytoplasmic and nuclear trafficking, and uncoating. Hence, understanding capsid dynamics is critical to improving virus efficacy. Protruding from the AAV capsid shell are N-terminal extensions with known roles in virus endosomal escape and nuclear trafficking, though little is known of these mechanistic details. Remarkably, in all human AAV serotypes and several other parvoviruses, we identified a highly-conserved, three residue serine/threonine (S/T) motif in the capsid N-terminus; the significance of which has been largely ignored. In this study, we investigate the functional significance of the AAV capsid N-terminal S/T motif in intracellular virus-host interactions. Our results show that the N-terminal S/T motif plays a key role for AAV transduction in serotypes 2, 4, 7, 8, and 9. Through alanine scanning mutagenesis, we determined the first serine residue of the AAV2 S/T motif has a critical role in transduction; mutating the serine to alanine decreases transduction efficiency 12.5-fold. Mutating all 3 serines in the motif to alanines virtually ablates transduction - 25-fold lower than wild-type (WT) AAV2- suggesting a synergistic effect between the serines. Assays for virus formation, internalization, cytoplasmic and nuclear compartmentalization, and capsid uncoating show no differences between WT and mutated vectors that can explain the decrease in transduction we observe with the S/T mutants. Remarkably, we observe a 3-fold decrease in viral transcripts of the triple-alanine substituted S/T virus. We are concurrently investigating a potential role of the S/T motif in exporting viral mRNA into the cytoplasm, possibly implicating the AAV capsid in a novel role downstream of uncoating and in gene expression. Sequence alignment of this N-terminal capsid region with other AAV serotypes reveals single amino acid variations between S/T motifs (AAV9 has an alanine (SSA), and AAV 4, 7 and 8 have threonines (SST) in the 3rd position of the S/T motif). We are currently investigating the functional relevance of the S/T motif in other serotypes by restoring the serine in the 3rd position and through alanine scanning. We have demonstrated that this short motif in the AAV capsid is amenable to manipulation without consequence on virus formation yet can result in striking changes in gene transfer. Taken together our data implicates the S/T motif is an important mediator of AAV transduction and gene expression.

664. Engineering Adeno-Associated Virus with a Self-Peptide for Immune-Avoidance

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As a gene therapy vector, adeno-associated virus (AAV) offers several advantages of high persistence, broad tropism, and biosafety. Unfortunately, activation of the immune response by the virus capsid is a significant barrier for effective and widespread use in a clinical setting. Most studies so far have examined the role of the adaptive immune response against AAV. In order for these adaptive responses to occur, the innate immune response is likely playing an initial role. For example, an early study by others demonstrated that AAV vectors are phagocytosed by macrophages *in vivo*. This uptake of AAV vectors by immune cells could lead to subsequent initiation of the adaptive immune response. In order to prevent AAV phagocytosis by immune cells, we investigated the use of the 'Don't Eat Me Signal'. Specifically, CD47-expressing cells bind with the immune cell SIRPα receptor to elicit a 'Don't Eat Me Signal' and to evade phagocytosis. Others have successfully synthesized and attached the SIRPα-binding bioactive region of CD47, named 'self-peptide' (SP), onto the surface of nanobeads to prevent phagocytic uptake. To decrease AAV phagocytosis, we generated AAV vectors with self-peptides inserted in the capsid (AAV-SP). Glycine-serine $(G4S1)_n$ linkers of different lengths were inserted in AAV-SP to relieve potential steric strain of SP insertion during capsid assembly. All mutants were structurally and functionally characterized for genomic titers, capsid SP and linker incorporation, and transduction efficiency. Results show the AAV capsid tolerates SP and glycine-serine linker insertion well. Notably, all mutants show dramatic decreases in phagocytic susceptibility compared to *wt* capsid *in vitro* in a THP-1 cell line, with displaying 10-fold decrease in immune cell uptake. Collectively, these results support the inclusion of the SP motif on the AAV capsid surface to inhibit immune cell phagocytosis. *In vivo* studies are underway to test immunological performance of the AAV-SP variants.

665. Harnessing the Activatable Peptide Display Behavior of Adeno-Associated Virus for Delivery of Peptides

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Peptide therapies have emerged as promising treatments for several diseases due to their powerful innate functions coupled with an attractive safety profile. However, the *in vivo* application of peptides is often unsuccessful because of their metabolic instability, rapid clearance, and poor cell entry. We believe that virus display of therapeutic peptides may remedy these challenges by incorporating peptides in a stable structure with a robust cell binding and uptake mechanism. To this end, we harnessed an innate activatable peptide display behavior demonstrated by adeno-associated virus (AAV) for delivery of peptides to targeted cells. AAV extrudes the N-termini of its longer capsid subunits as a response to the endosomal environment, facilitating endosomal escape and nuclear trafficking. We developed truncations of these longer capsid subunits, specifically the VP2 subunit, and combined them with the shorter native capsid component (VP3) into mosaic capsids. Through characterization of these virus nanoparticles (VNPs), we determined that capsid mosaicism can be used to tune the degree of activatability from an 'always on' constant peptide display (aka 'brush-like' peptide display) to an 'activatable' peptide display behavior. The panel of VNPs developed exhibits a spectrum of these behaviors, with higher levels of peptide display (both in absolute and in fold change post-activation) than the wt AAV capsid. We are now building upon this platform technology for the cellular delivery of therapeutic peptides. As a first pass, we are incorporating cytotoxic peptides into the VNPs in both the brush-like and activatable configurations. We are incorporating cytotoxic peptides that act at the cell surface into VNPs exhibiting brush-like display, and cytotoxic peptides that act intracellularly into activatable VNPs; thus taking

advantage of the unique display properties of our panel to best deliver peptides to their site of action. The engineered VNPs will be tested in both *in vitro* and *in vivo* models to determine if peptide delivery is improved over free peptide treatment. In the future, our engineered AAV vectors may enable the co-delivery of bioactive peptides/proteins and nucleic acids in a highly efficient manner.

Preclinical Approaches in Gene transfer to the Central Nervous System

666. Rett Syndrome Gene Therapy Improves Survival and Ameliorates Behavioral Phenotypes in MeCP2 Null

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Rett syndrome is a devastating progressive neurodevelopmental disorder, affecting approximately 1 in 10,000 girls. Rett patients experience loss of achieved developmental milestones, including speech and motor function, beginning at 6-18 months of age. Patients often survive for 40-50 years, but experience lifelong severe developmental disability requiring intense supportive measures, and 24/7 care. This produces a significant emotional and financial burden on patients and their families. Rett syndrome is caused by mutations in the X-linked methyl-CpG binding protein 2 (*MeCP2*) gene, encoding a ubiquitous transcription factor with activating and repressing functions for thousands of genes in the brain. Recent studies using rodent models have demonstrated that re-expression of *MeCP2* ameliorates Rett-syndrome-like phenotypes, including decreased survival and abnormalities in motor activity. Thus, we believe that gene replacement therapy is a promising therapeutic strategy for this disease. To this end, we have generated a self-complementary adeno-associated virus serotype 9 vector expressing the human *MeCP2* gene under the control of a truncated *MeCP2*-promoter (scAAV9.MeCP2). This promoter maintains parts of the endogenous regulatory elements to ensure appropriate expression levels of our construct. We determined the efficacy and safety of this vector in male mice (MeCP2-null and wild-type) and male non-human primates (NHPs; *Macaca fascicularis*). Mice were injected intracerebroventricularly with multiple doses, and behavior (e.g., rotarod and open field) and survival were monitored. Weight gain as well as blood and liver parameters of five juvenile NHPs were monitored after lumbar intrathecal injection of scAAV9.MeCP2. To date, three NHPs have been sacrificed for transgene expression and pathology evaluation. We found that all doses of scAAV9.MeCP2 tested in MeCP2-null mice increased survival and rescued behavioral symptoms. The most promising dose increased the median lifespan from 66 to 315 days. No toxicity was observed in wild-type mice. In NHPs, weight, blood parameters and liver enzymes remained normal up to the time of abstract submission (18 months post treatment). No indications of pathology were found in the NHPs that were sacrificed at 5 weeks post injection. Overall, our results show good expression of MeCP2 throughout the whole central nervous system after a single injection. In MeCP2-null mice, treatment more than quadrupled the lifespan, indicating high therapeutic potential. We will continue to examine the safety of our vector in treated mice as well as NHPs. Completion of this work will be followed by additional mouse and NHP safety studies in order to support the initiation of a human clinical trial in the near future.

667. From Bench to Bedside: Gene Therapy for Batten (CLN6) Disease

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Batten Disease is a fatal, neurodegenerative lysosomal storage disorder with multiple causative genes and a wide range of disease severities. Mutations in the ceroid-lipofuscinosis neuronal 6 (CLN6) gene mainly affect children between 18 months and 8 years of age. The disease is characterized by seizures, progressive dementia and loss of visual and motor functions, ultimately leading to death within the first 15 years of life. Unfortunately, there is no cure or therapeutic treatment for CLN6-Batten disease. The disease is caused by mutations causing absence or reduced abundance of CLN6 protein, making gene therapy a promising therapeutic strategy. The objective of this study was to determine the feasibility of adeno-associated vector serotype 9 (AAV9) mediated CLN6 expression as a viable gene therapy for CLN6-Batten disease. We developed a scAAV9 vector expressing the human CLN6 (hCLN6) gene under the control of a chicken β-actin (CB) promoter. To determine the efficacy, scAAV9.CB.CLN6 was administered by a single, postnatal intracerebroventricular injection in CLN6 mice. Mice were assessed for histopathological, behavioral and survival changes. The one-time treatment resulted in drastic reduction of accumulated autofluorescent storage material and ATP synthase subunit C (both hallmarks of the disease) by as early as one month of age. There was a significant improvement in motor performance (rotarod assessment) in scAAV9.CB.CLN6 treated animals starting at 8 months of age compared to uninjected CLN6 mice. The treatment also resulted in significant improvement of learning and memory deficits in CLN6 mice demonstrated by improved performance of scAAV9.CB.CLN6 treated mice in the Morris water maze test. Importantly, while all untreated CLN6 mice died between 12-14 months of age, scAAV9.CB.CLN6 administration significantly extended the median survival beyond 22 months of age and all of the histopathological, behavioral and cognitive parameters continued to improve throughout the lifespan of the treated mice. To our knowledge, this is the longest survival extension reported in this mouse model to date. To further translate this approach towards clinic, we dosed three 4-year old Cynomolgus Macaques with scAAV9.CB.CLN6 by intrathecal lumbar injection

and monitored them for up to 6 months post injection. No adverse effects or pathology were observed, while high levels of transgene expression were found throughout the brain and spinal cord of all three animals. Collectively, this study provided a strong foundation for the currently ongoing first in-human phase I clinical trial for intrathecal administration of scAAV9.CB.CLN6 in CLN6-Batten disease patients.

668. A Novel Brain-Directed Hematopoietic Stem Cell (HSC) Gene Therapy Approach Provides Unique Therapeutic Benefit to the Mouse Model of Infantile Neuronal Ceroid Lipofuscinosis (CLN1)

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Infantile neuronal ceroid lipofuscinosis (INCL), caused by defects in CLN1 gene coding for palmitoyl protein-thioesterase-1 (PPT1), is one of the most severe forms of NCLs, leading to progressive vision loss, dementia, epileptic seizures and loss of motor coordination, culminating in premature death. Achieving high levels of the wild type enzyme in the central nervous system (CNS) is a key factor for successful correction of the pathology. To this aim, we generated a local source of the wild type PPT1 in INCL mouse brain by a novel, brain-directed HSC gene therapy approach. To this goal we exploited i) a lentiviral vector expressing supra-physiological levels of the wild type human codon-optimized PPT1 (up to 15-fold), and ii) a novel delivery route for HSC gene therapy, namely the injection of the transduced HSCs in the brain lateral ventricles (intracerebroventricular - ICV) to foster the CNS engraftment of HSC-derived cell progeny and the associated therapeutic benefit. In particular, we compared the efficacy of a standard HSC gene therapy approach based on intravenous (IV) delivery of transduced Ppt1-/- HSCs, with an ICV only approach or a combinatorial (IV+ICV) transplant strategy. These approaches were tested in young adult, 6-8 weeks old, busulfan myeloablated Ppt1-/- recipients. Robust engraftment of donor-derived cells, measured in peripheral blood, was observed in all groups receiving gene corrected HSCs IV, while as predicted the transduced cells remained confined to the CNS in the ICV only group. All the tested treatments resulted in a significant increase of survival and phenotype amelioration in the transplanted mice at comparison with mock-transplanted Ppt1-/- mice ($p<0.01$). Ppt1-/- mice receiving the transduced HSCs both IV and ICV displayed the best treatment outcome, with complete prevention of disease manifestations and survival extension up to over 60 weeks of age at the most recent time-point of the on going observation (average survival of untreated animals is 34±0.9weeks). The other groups, and in particular the ICV only group, showed a significantly delayed onset of neurologic symptoms that may underline the involvement of the peripheral nervous system pathology, which is not targeted by the ICV cell delivery route. Analyses are on-going to correlate clinical findings with brain PPT1 activity, transduced-cell chimerism and histological readouts (quantification of storage, neuronal survival and markers of neuroinflammation). These preliminary results and the clinical benefit observed in the treated animals are remarkable and unique since they have been observed upon treatment of young adult 6-8 weeks old animals, in which storage and histopathological alterations are already accumulating. Existing literature and other thus far successful gene therapy approaches have shown a similar benefit upon treatment administration to newborns or pups. Thus, the clinical benefit reported by our study is uniquely promising and provides for compelling evidence of feasibility and efficacy of a novel brain-directed HSCs gene therapy approach for treating INCL, paving the way for clinical development of this approach for the treatment of this devastating disease and possibly other neurodegenerative conditions.

669. AAV-Mediated CYP46A1 Gene Therapy as a Strategy to Counteract Huntington's Disease

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Defects in brain cholesterol metabolism in the adult brain are associated to neurodegenerative diseases, such as Alzheimer's and Huntington's diseases (HD). In HD, defects in cholesterol homeostasis include a general perturbation in the expression of cholesterol biosynthesis enzymes. 24-S-hydroxycholesterol (24OH-Chol), the catabolite of cholesterol metabolism, is reduced in the plasma of HD patients. CYP46A1 levels, the rate-limiting enzyme, which catalyzes the production of 24OH-Chol in neurons, are declined in the striatum of HD patients and HD mouse models. CYP46A1 plays major roles in activating brain cholesterol turnover, therefore increasing the mevalonate pathway, with beneficial effects on synaptic plasticity. Restoring CYP46A1 expression in vivo by adeno-virus-mediated (AAV-CYP46A1) delivery in the striatum of two HD mouse models (R6/2 and ZQ175) results in the reduction of huntingtin aggregates, hallmarks of pathology, increased recovery of neuronal markers associated to noteworthy improvement in motor abilities. Moreover, cholesterol biosynthesis pathway is restored in the targeted brain regions, leading to a normalization of cholesterol, desmosterol, lanosterol and 24OH-Chol levels in these two HD mouse models. Furthermore, we demonstrate that AAV-CYP46A1 corrects the BDNF/TrkB pathway that is dramatically impaired in HD, as well as, vesicular transport. Towards clinical evaluation in HD patients,

dose-responsive studies in mice and translational steps in non-human primates were performed, demonstrating the efficacy and tolerance of AAV-CYP46A1 delivery in the striatum. Therefore, we propose a phase I/II clinical application to evaluate the efficacy and safety of a single AAV-CYP46A1 administration in the striatum of HD patients at early stages of HD progression.

670. Virally-Mediated Dopamine Autoreceptor Expression Blocks Levodopa-Induced Dyskinesia Development by Inhibiting False Neurotransmission of Serotonin Neurons

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Parkinson's disease (PD) is a multisystem disorder characterized by the loss of dopamine (DA) signaling in the striatum due to the death of neurons in the substantia nigra, resulting in the hallmark motor deficits. The current 'gold standard' treatment, levodopa (L-DOPA), has been in use since the late 1960s as a DA replacement therapy to ease motor deficits. Unfortunately, chronic treatment with L-DOPA leads to development of L-DOPA-induced dyskinesias (LIDs), a series of debilitating and disrupting motor symptoms including hyperkinesia, dystonia, and chorea. While the mechanisms that underlie LIDs are a multifaceted process, a growing body of research suggests that the dysregulation of DA synthesis and its release as a false neurotransmitter by striatal serotonin terminals derived from dorsal raphe neurons may play a pivotal role in dyskinesia genesis. Serotonin neurons can convert L-DOPA into DA, but do not express the regulatory mechanisms required for physiological modulation of DA signaling. This would lead to an unregulated and excessive release of DA into the DA-depleted, hypersensitive striatum, promoting LID development. Preclinical studies have utilized serotonin receptor agonists to treat LIDs by dampening neurotransmitter release from these neurons, but promising animal data has been not translated in large scale clinical trials. Importantly, while there is an abundance of convincing clinical and preclinical evidence supporting a role of serotonergic input in LID expression, there is no direct evidence that lack of autoregulation underlies dysregulated DA release from serotonergic neurons and impacts LID formation. In the present study, we aimed to determine if LIDs could be prevented by ectopically expressing DA regulatory elements—specifically the D2 autoreceptor ($D2R_s$)—in dorsal raphe serotonin neurons. Fischer344 rats were rendered unilaterally parkinsonian with 6-hydroxydopamine (6-OHDA), and subsequently received either rAAV2/9 expressing $D2R_s$ or GFP to the dorsal raphe via stereotaxic injection. When subjected to chronic L-DOPA treatment (2-12mg/kg), animals overexpressing $D2R_s$ showed a complete resistance to LID development, whereas rAAV-GFP controls showed robust LID expression. *In vivo* microdialysis showed that rAAV-$D2R_s$ treatment in raphe neurons reduced DA efflux in the striatum with no effect on serotonin release as compared to rAAV-GFP controls. These data provide the first direct evidence of abnormal DA release from serotonin neurons in a parkinsonian model, and show that these neurons can regulate DA signaling utilizing a novel rAAV-$D2R_s$ gene therapy approach. Importantly, rAAV-$D2R_s$ treatment did not change the anti-parkinsonian efficacy of L-DOPA as shown in the cylinder and adjusting steps tests. Taken together, these data provide strong evidence that dysregulation of DA signaling by dorsal raphe serotonin neurons is a critical event in LID development. This direct evidence of false neurotransmission and its prevention with rAAV-$D2R_s$ gene therapy confirms the serotonin hypothesis in LIDs and a modulation of serotonergic neurons as a potential therapeutic approach for patients.

671. Preclinical Development of Gene Therapy for Niemann Pick Disease Type A

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Lysosomal Storage Diseases (LSD) are caused by a variety of recessive gene mutations that lead to a major impairment in lysosomal function. Niemann-Pick disease (NPD; about 1 in 7700 live births) is emblematic for a number of LSD. Two different forms (NPD-A and NPD-B) are caused by mutations in SMPD1 gene (Acid Sphingomyelinase or ASM). NPD-A is associated with a profound neurological deficit, and this difference is most probably due to the different levels of residual enzyme activity in various tissues that NPD-A and NPD-B present. Patients with NPD-A have extremely low levels of ASM (less than 5% of normal) whereas those with NPD-B have approximately 10% of the normal level of ASM. The fact that quite low residual levels of ASM in NPD-B patients prevent the neurological symptoms of Type A disease suggests that replacement gene therapy will not require massive overexpression of the ASM transgene. To date, enzyme replacement therapy has proven successful to treat NPD-A peripheral symptoms but not brain pathology. Previous studies in our laboratory with MR-guided direct brain infusions of AAV2 carrying a correct copy of the ASM gene in non-human primates (NHP) showed that focal overexpression of sphingomyelin generates significant local neurotoxicity and motor impairment in all the animals treated. Based on these studies and our recent work in CSF delivery, we think that lower levels of transduction with a more global distribution could be an adequate therapeutic approach. In the present study, we evaluated the safety of cerebellomedullary cistern (CM) injection of AAV serotype 9 encoding human ASM (AAV9-hASM) in NHP and its therapeutic benefit in the ASM*ko* mouse model. Our data revealed that CM injection of AAV9-hASM in NHP resulted in transgene expression in brain cells along the anteroposterior axes, including cerebellum, without significant inflammatory response in all survival times evaluated. ASM*ko* mouse experiments revealed that vector dose tested was efficacious, showing motor and memory skills improvement, with no sphingomyelin accumulation and neuronal death. Our results support CM injection for future AAV9-based clinical trials in NPD-A as well as other monogenic lysosomal storage brain disorders.

672. Rescue of Central and Peripheral Neurological Phenotype in a Mouse Model of Friedreich's Ataxia by Intravenous Delivery of AAV Frataxin with a Novel Capsid

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Friedreich's Ataxia (FA) is caused by an intronic GAA expansion in the frataxin gene leading to significantly decreased expression of frataxin (FXN), an essential protein involved in mitochondrial function. Most patients initially develop difficulty in walking and loss of balance due to the degeneration of large proprioceptive neurons in the peripheral dorsal root ganglia (DRG). Subsequently trunk and arm function decline and increasing spino-cerebellar neuronal impairment develops along with degeneration of the dentate nucleus of the cerebellum. Patients become wheelchair bound and incapacitated, leading to a reduced average life-span of about 40 years of age. To model the selective nature of neuronal loss in FA, a transgenic mouse was created in which FXN expression is abolished via the Cre-Lox system in parvalbumin expressing cells (Pvalb FXN cKO mice), including large sensory neurons in the DRGs and cerebellar neurons. The mice showed progressive loss of proprioceptive sensory function and progressive ataxia within weeks after birth. We previously treated Pvalb FXN cKO mice intravenously with a novel adeno-associated virus capsid (AAVVOYF2) carrying a transgene for cynomolgus (primate) frataxin with an HA-tag (AAVVOYF2-FXN-HA), together with intracerebral dosing of AAVrh10-FXN-HA. This treatment prevented central and peripheral disease progression after symptom onset in Pvalb FXN cKO mice (Puccio et al., ESGCT Annual Congress, Berlin, October 17-20th 2017, Poster 107). In the current study, we delivered AAVVOYF2-FXN-HA solely intravenously post-symptomatically in Pvalb FXN cKO mice at 7.5 weeks of age. Three dose levels were evaluated for efficacy on sensory and motor function by electromyogram, notched bar walking, rotarod and string hanging assays. In all tests, AAVVOYF2-FXN-HA rapidly reduced disease progression in a dose-dependent manner compared to vehicle- treated Pvalb FXN cKO mice. Our long-term analysis (52 weeks) demonstrated a complete lack of disease progression in the treated mice, including the CNS phenotype. These new results support the study of intravenous frataxin gene therapy with novel AAV capsids for treating both central and peripheral neurological disease in FA.

Metabolic, Storage, Endocrine, Liver and Gastrointestinal Diseases

673. Engineering the Hematopoietic System for Lysosomal Storage Disorders: Correction of Mucopolysaccharidosis Type I Using Genome-Edited, Human Hematopoietic Stem and Progenitor Cells

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Lysosomal storage diseases (LSDs) are a large group of genetic disorders, many of which lack effective treatments. MPSI is an LSD caused by a deficiency in α-L-iduronidase (IDUA) that results in a progressive multi-systemic disorder that encompasses neurologic, musculoskeletal, and cardiorespiratory deterioration. Current interventions for some LSDs (including MPSI) are enzyme replacement therapy and allogeneic hematopoietic stem cell transplantation. Both have limited efficacy and at best slow the progression of these diseases. **A potentially safer, more effective approach is to engineer the patient's own hematopoietic system to secrete high levels of enzyme via autologous transplantation of genome-edited cells.** Additionally, a genetic strategy where lysosomal enzymes are targeted to a safe harbor locus, could constitute an adaptable "one size fits many" approach that is independent of specific lysosomal enzymes or patient mutations. We describe a highly efficient genome editing strategy to target IDUA into the *CCR5* safe harbor locus in human CD34$^+$ hematopoietic stem and progenitor cells (HSPCs). Using sgRNA/Cas9 ribonucleoprotein and adeno-associated viral vector delivery of a homologous donor we achieved median HSPC modification frequencies of 31.2% (IQR 26.4-34.3) with expression cassettes containing IDUA and yellow fluorescent protein, 47.4% (40.8-79.9) with the IDUA coding sequence alone in cord blood-derived HSPCs, and 21.1% (15.5-24.1) and 30.2% (28.3-32.5) from adult peripheral blood-derived HSPCs. Expression driven by the spleen focus-forming virus and phosphoglycerate kinase promoters resulted in supra-physiological levels of IDUA expression of 240-fold and 25-fold respectively, which persisted for at least 30 days in culture. Long-term and serial engraftment studies in NOD-scid-gamma (NSG) mice demonstrated modification of cells with long-term repopulating capacity, albeit at lower efficiency than hematopoietic progenitors. The median percentage of hCD45$^+$/HLA$^+$ cells in the bone marrow was 1.8% (min-max: 0.16-96%) at week 16 and 3.6% (0.9-91%) after CD34 selection and serial transplantation. Neither the genome editing process nor IDUA expression affect the modified cells' potential to differentiate into multiple hematopoietic lineages *in vitro* or *in vivo*. The capacity of the modified cells to improve the symptoms of the disease was demonstrated using an MPSI mouse capable of engrafting human cells. MPSI-NSG mice were established by knocking-in a mutation analogous to the W402X mutation, common in patients with severe MPSI, in the NSG background. These mice have expected tissue accumulation and urinary excretion of glycosaminoglycans (GAGs) as well as phenotypic features of progressive dysmorphism, bone dysplasia, and neurobehavioral deficits as described in

immunocompetent MPSI mice. Transplantation of the IDUA-HSPCs into MPSI-NSG mice resulted in increased IDUA activity to 1.2, 3.2, 1.5, and 3.1% of normal in serum, liver, spleen, and brain respectively (compared to undetectable prior to transplantation). Transplantation also resulted in decreased urine and tissue GAGs. The amount of biochemical correction was correlated with the percent human engraftment. Significant improvements were also observed in spontaneous locomotion, memory and anxiety deficits. **Our studies support the use of genome edited human HPSCs for the treatment of MSPI using autologous transplants. The safe harbor approach constitutes a flexible platform for the expression of other lysosomal enzymes, thereby establishing a potential new paradigm for delivering enzymes for the treatment of other LSDs.**

674. Efficacy and Safety of Long-Term Prophylaxis in Severe Hemophilia a Dogs Following Liver Gene Therapy Using AAV Vectors: A 10 Year Follow-Up Report

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Hemophilia A (HA) is an X-linked bleeding disorder characterized by a deficiency in clotting factor VIII (FVIII). Preclinical studies in HA mice, HA dogs and non-human primates supported the current clinical trials for adeno-associated viral (AAV) gene delivery for HA that have reported therapeutic FVIII activity (FVIII:C) up to 1 year (Rangarajen 2017). Our preclinical studies of AAV-canine FVIII (cFVIII) delivery in HA dogs utilized 2 delivery approaches: (1) co-administration of 2 AAV vectors encoding separate cFVIII heavy and light chains driven by the thyroxine binding globulin (TBG) promoter (Two chain approach)(TC) or (2) delivery of cFVIII as a single chain driven by the human alpha-1 anti-trypsin (hAAT) promoter (Single chain approach)(SC)(Sabatino 2011). We demonstrated that both strategies were efficacious, prevented >90% of spontaneous bleeding episodes, and had no evidence of toxicity. In addition, there was no evidence of immune responses to FVIII. We now report the long-term follow-up of these HA dogs treated with AAV-cFVIII for as long as 10 years before terminating the study to obtain additional information about the safety and efficacy of this approach. To address long-term efficacy, we measured cFVIII:C (Coatest SP4 FVIII). For dogs receiving the TC approach at the high dose, the mean cFVIII activity was 4.4% and at the low dose was 4.9% ± 2.1. The dogs receiving the SC approach at the high dose had a mean cFVIII activity that was 6.3% and at the low dose was 1.7%. No anti-cFVIII antibodies were detected for the duration of the study. Ten years following the treatment of severe HA dogs with AAV-mediated liver-directed gene therapy, we observed long-term expression of therapeutic levels of FVIII using either a TC or SC delivery approach with no immune response to FVIII. Stable FVIII expression was maintained for 7 of the dogs over the course of the study, however, 2 dogs (Linus and M50) had a gradual increase in FVIII:C that began ~3 yrs after vector administration. Liver function tests, serum alpha-fetoprotein concentrations, fibrinogen levels as well as liver pathology did not suggest any evidence for altered liver function or tumor development. Clinically there was no evidence of malignancy and no tumors were detected at the time of necrospy. Based on the current findings, it is unlikely that the increase in FVIII expression was due to an integration event. Interestingly, Linus (TC) and M50 (SC) were treated with different delivery approaches that each utilize a different liver specific promoter but both were delivered with AAV8. Interestingly, this rise in transgene expression after AAV administration was not observed in long-term follow-up of hemophilia B dogs (Niemeyer 2009) suggesting that this may be specific to FVIII. Further studies are underway to determine the mechanism of increased FVIII expression, which we hypothesize may be related to the clearance of the FVIII protein. Together these studies demonstrate the longest sustained FVIII expression in a large animal HA model with no evidence of liver toxicity as long as 10 years following AAV administration. These data provide important safety information that further supports the clinical development of liver-directed AAV-mediated gene therapy for HA.

AAV-cFVIII Treated Hemophilia A Dogs

FVIII Delivery Approach	AAV Vector Dose	HA Dog	Yrs Evaluated	% cFVIII Activity (Mean)	% cFVIII Activity (Final)
Two Chain	$1.25x10^{13}$ vg/v/kg	F24	2.2	5.7 ± 2.3	2.7
		Woodstock	8.8	3.1 ± 1.5	4.6
	$6x10^{12}$ vg/v/kg	J60	9.5	8.1 ± 4.0	4.5
		Linus	10.4	5.9 ± 4.6	11.3
		H19	8.8	0.9 ± 0.7	2.5
Single Chain	$4x10^{13}$ vg/kg	M06	2.3	7.4 ± 2.6	9.4
		M50	8.8	5.1 ± 3.3	13.8
	$2x10^{13}$ vg/kg	L51	6.1	0.8 ± 0.6	2.9
		M66	6.3	2.6 ± 1.3	3.7

675. A Phase 1 / 2 Clinical Trial of Systemic Gene Transfer of rAAV9.CMV.hNAGLU for MPS IIIB: Safety, Tolerability, and Preliminary Evidence of Biopotency

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Sanfilippo syndrome type B (mucopolysaccharidosis type IIIB), a lysosomal storage disorder due to mutations in the *NAGLU* gene, gives rise to intracellular glycosaminoglycan accumulation and results in cellular dysfunction and death. Although multisystemic, central nervous system (CNS) findings predominate. An open-label, dose-escalation Phase 1/2 gene transfer trial was initiated using intravenous administration of a rAAV9.CMV.hNAGLU vector with tropism to both the CNS and relevant somatic tissues. The primary outcome measure

of the trial is safety; secondary outcome measures of efficacy at 6 and 12 months post-dosing include CSF and urine type-specific heparan sulfate (HS) fragment levels, liver and spleen volumes by MRI, CSF and plasma NAGLU activity levels, cognitive function (by the Leiter International Performance Scale-Revised and the Mullen Scales of Early Learning), and parental assessment of adaptive behavior (the Vineland Adaptive Behavior Scale, 2nd edition). A single subject has been enrolled in cohort 1 (2 X 10^{13} vg/kg); the gene transfer was well-tolerated without significant adverse events, and enrollment is continuing. All subjects receive oral prednisolone from the day prior to gene transfer until at least 60 days after transfer; in the first patient, serum transaminases have remained within the pre-treatment range, and no significant T-cell immune responses to the capsid or transgene product have been noted. Normal levels of plasma NAGLU enzyme activity have been detected at days 7, 14 and 30 post-transfer, liver volume diminished as measured by MRI, and reductions in CSF, urine and plasma heparan sulfate levels have been observed. This study is supported by Abeona Therapeutics.

676. Correction of Advanced Pompe Phenotype in Mice with AAV Liver Gene Transfer of Secretable GAA

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Pompe disease is a neuromuscular disorder caused by mutations in the gene which encodes for the lysosomal enzyme acid α-glucosidase (GAA). GAA converts lysosomal glycogen to glucose, and its deficiency leads to pathologic glycogen accumulation. Infantile-onset Pompe disease (IOPD) is very severe and affects newborn infants with severe cardio-respiratory defects that lead to premature death. Late-onset Pompe disease (LOPD) presents later in life with progressive muscle weakness and deterioration of respiratory function. Enzyme replacement therapy (ERT) is the only available treatment for Pompe disease and, in spite of being a life-saving treatment for IOPD patients, it suffers from several shortcomings, including limited efficacy and high immunogenicity. In LOPD patients, ERT has been shown to stabilize rather than improve the disease phenotype. We recently reported full rescue of the Pompe phenotype in symptomatic four month-old $Gaa^{-/-}$ mice using AAV liver gene therapy with a secretable form of GAA (*Puzzo, Colella et al., Sci Trans Med, 2017*). To assess whether this approach could result in therapeutic efficacy in $Gaa^{-/-}$ mice with advanced pathology, we treated nine month-old $Gaa^{-/-}$ mice with an AAV8 encoding for secretable GAA and followed the animals for 9 months thereafter. Previous gene therapy studies aiming to correct Pompe disease in old $Gaa^{-/-}$ mice failed to restore normal glycogen levels in the central nervous system, or skeletal and cardiac muscle, and also reported transient or no improvements in disease phenotype (*Raben et al., Mol Ther, 2002*; *Ziegler et al., Hum Gene Ther, 2008*; *Sun et al., J Gen Med, 2009*). In our study, nine months after treatment, AAV-treated $Gaa^{-/-}$ mice showed high and sustained circulating levels of GAA activity which correlated with a complete clearance of glycogen in cardiac and skeletal muscle, and a ~30% glycogen reduction in the nervous system. Moreover, we observed an amelioration of respiratory symptoms and a complete normalization of muscle strength in the AAV-treated mice cohort which was also supported by the normalization of autophagic buildup in skeletal muscle. We further observed that AAV-GAA gene transfer corrected mitophagy (mitochondrial specific autophagy) which plays pivotal roles in cellular bioenergetics homeostasis. These results in old $Gaa^{-/-}$ mice with advanced disease state shed light on the reversibility of the disease phenotype in the Pompe mouse model via liver gene transfer of secretable GAA.

677. Molecular Characterization of Hematopoietic System Reconstitution in Metachromatic Leukodystrophy Patients Following Hematopoietic Stem Cell Gene Therapy

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Metachromatic leukodystrophy (MLD) is an inherited lysosomal storage disease caused by arylsulfatase A deficiency, lethal in early childhood after disease onset. We are following a lentiviral vector based hematopoietic stem cell (HSC) gene therapy clinical trial at SR-Tiget. For 20 treated patients (plus 2 additional patients treated via early access programs) we have characterized the hematopoietic reconstitution in vivo, up to 6 years follow-up, at a molecular level using vector integration sites (IS) as clonal identifiers. We used a canonical and a novel PCR protocol for the unbiased and quantitative integration site (IS) retrieval in CD34+, myeloid and lymphoid cells purified at different time points after therapy from bone marrow and/or peripheral blood. From each patient, we retrieved from 6,613 to 61,820 IS, many of which persisted long term and were shared across lineages. For the assessment of the safety of the treatment, we did not observe clonal dominance events, nor bias to integrate near cancer genes and no common insertion sites generated by genetic selection. IS were shared among HSC and differentiated cells, indicating that multilineage reconstitution of genetically modified cells has occurred. We estimated the number of HSC by mark-and-recapture statistics using short-lived cells as a surrogate of HSC and the results showed that at earlier time points (up to 9 months) the population size was more than 26,000 cells that then progressively stabilized to around 8.000 from 9 months post-transplantation, suggesting that the initial waves of reconstitution are

sustained by short-lived progenitors. The analysis of clonal dynamics of the hematopoietic reconstitution in different lineages showed that circulating lymphoid cells were oligoclonal at early time-points and progressively switched to polyclonal after 6 months, whereas myeloid cells were polyclonal since the first time points. Our data indicate that the treatment results in a highly diversified polyclonal and multilineage reconstitution of hematopoiesis without signs of genotoxicity.

678. Therapeutic Effect of Neonatal Gene Transfer and Bone Marrow Transplantation in Neuronopathic Gaucher Disease

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Lack of effective treatment for neuronopathic Gaucher Disease (nGD) results from the difficulty of therapeutics to penetrate the blood-brain-barrier. For monogenetic diseases, hematopoietic stem cell transplantation in the neonatal period (nHSCT) may provide a novel approach to providing sustainable/therapeutically effective levels of the deficient enzymes/proteins, thus preventing disease progression. Here, such potential benefits of nHSCT and nHSCT-mediated gene therapy to treat nGD at newborn stage were evaluated using a mouse model of nGD (4L;C*) that mimics human nGD variants. HSC transplantation with cells derived from GFP transgenic mice that express wild type levels of acid ß-glucosidase (GCase) showed >80% engraftment in platelets, red blood cells and leukocytes. FACS analyses showed that donor-derived myeloid cells (BMDM, $CD45^+CD11b^+$) repopulated in the myeloid compartment of the recipient brain ~2-months after nHSCT. GFP^+ cells accounted for 20% of meningeal and choroid plexus macrophages (i.e., CNS ϕ cells, $CD11b^+CD45^{high}MHC^{high}$). Immunofluorescence staining revealed that the (repopulated) BMDM cells are mainly distributed at thalamus, brain stem and midbrain. Although nHSCT in 4L;C* mice showed sub-physiological GCase activity in the brain and normalized levels in liver, WT HSCs failed to extend the lifespan of 4L;C* mice or mitigate neurological phenotype. To improve the efficacy, HSC cells were transduced using lentiviral vector to overexpress GCase as well as the mCherry marker gene under the transcriptional control of a spleen focus-forming virus (SFFV) promoter. Neonatal transplantation of such re-engineered HSC cells (nGT) provided potent therapeutics depots in busulfan conditioned 4L;C* mice. Two-months post transplantation, the frequency of transgene (mCherry) expressing cells (as determined by FACS analysis) varied among three recipients, ranging from 0.2%-72% in platelets, 11%-96% in leukocytes and 31%-99% in red blood cells. The 4L;C*/nGT mice exhibited elevated GCase activity in plasma (mean of 26-fold of normal) and liver (3-fold), and higher-than-untreated levels in the brain, which were also associated with gene transfer efficiency (mCherry%). Compared to untreated 4L:C* mice, the lifespan in 4L;C*/nGT significantly extended from an average of 60d to 67d, and age-of-onset for body-weight decline was delayed from 42d to 47d. Importantly, nGT-4L;C* mice exhibited significant improvement toward normal walking pattern by gaiting analysis, indicating amelioration of sensorimotor dysfunction. Additional efforts are underway to further evaluate the efficacy of neonatal gene therapy for the treatment of nGD. These findings demonstrate the feasibility of neonatal BMT combined gene therapy for nGD, which may provide an option to treat such neuronopathic patients at their early stage of life.

679. Isolation of an Adeno-Associated Virus 8 Variant with Improved Properties for Liver Gene Therapy

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AAV vectors have shown efficacy in the clinic for disorders such as hemophilia although curative therapies for a broader range of liver metabolic diseases will require improved vectors. Our goal in developing better hepatotropic vectors is to achieve more efficient transduction across the portal axis that is stable over time while maintaining high yields during manufacturing and favorable safety profiles. We performed mutagenesis on surface-exposed residues in hypervariable region 8 of AAV8 to create a library of capsids that were subjected to sequential rounds of selection in various models. The library initially was administered intravenously into FRG mice that harbored a liver reconstituted with human hepatocytes. At necropsy, AAV capsid DNA was recovered from human hepatocytes isolated from liver, and used to generate a new AAV library. This selection was repeated a second time in the mouse xenograft model. The final round of selection was conducted in a rhesus macaque injected with the AAV library covered from the second mouse liver xenograft selection. A capsid that demonstrated several logs of enrichment through the three cycles of selection as determined by NGS was isolated and further characterized. This AAV8 variant called AAVG3 demonstrated improved transduction to liver as compared to AAV8 following intravenous infusion in C57BL/6 mice and rhesus macaques. The pattern of transduction in mouse liver was evenly distributed through the portal axis while AAV8 transduced hepatocytes primarily around the portal triad. AAVG3 was manufactured at yields equal to or higher than that achieved with AAV8. AAVG3 may provide an advantage over AAV8 in the treatment of liver diseases.

Preclinical Pharmacology and Toxicology Studies and Assessment of Gene Therapy in Large Animal Models

680. Evaluation of Tolerability and Immunogenicity of EDIT-101 Following Subretinal Injection in Non-Human Primate

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Leber Congenital Amaurosis (LCA) type 10 is an early-onset retinal degeneration caused by mutations in the CEP290 gene. Currently, there's no approved treatment for LCA10. AAV-mediated gene replacement therapy is not feasible because the large size of the CEP290 cDNA (~7.5kb) exceeds the packaging capacity of AAV. EDIT-101 was developed to overcome this limitation and achieve a potentially functional cure. EDIT-101 is an AAV5 vector with targeted expression of S. aureus (Sa) Cas9 and CEP290-specific gRNAs in photoreceptor cells. The SaCas9/gRNAs-mediated deletion of the common CEP290 IVS26 c.2991+1655 A>G mutation was previously shown to restore the normal CEP290 expression in LCA10 patient-derived fibroblasts homozygous for the IVS26 mutation. EDIT-101 was also shown to achieve therapeutically relevant levels of targeted editing in human CEP290 IVS26 knock-in mice. To support the clinical development of EDIT-101, we evaluated the tolerability and immunogenicity following a single dose of either EDIT-101 or the surrogate NHP vector targeting cynoCEP290 gene in cynomolgus macaques. Both AAV5 vectors were produced at development scale with processes similar to that of clinical manufacture and formulated in BSS/Pluronic F-68 buffer. NHP was chosen because the retinal structure is anatomically comparable to human. Submacular injection used the clinically intended device and 2 stage approach resulting in an approximately 10-12 mm wide subretinal bleb following the dose of 100 uL of the NHP vector (7E+11 vg/mL), EDIT-101 (1E+12 vg/mL), or BSS/P. The surgery procedure, formulation and vectors were well tolerated based on clinical examinations including indirect ophthalmoscopy, slit-lamp biomicroscopy, and intraocular pressure. The scoring of the anterior and posterior changes was based on modifications of the Standardization of Uveitis Nomenclature (SUN), Hackett-McDonald, and SPOTS systems and performed by a board-certified veterinary ophthalmologist at pre-study, Days 3, 8, 15 and up to week 13. Electroretinography at pre-study and weeks 6 and 13 will be measured to assess any potential functional adverse effect. To evaluate any potential impact of pre-existing humoral and cellular immunity to AAV5 and SaCas9 protein on the efficacy and safety of the therapy, we developed Luminex-based ADA assays for measuring AAV5- and SaCas9-specific antibodies (Ab), and a SaCas9-specific T cell ELISPOT assay. In a specific colony of cynomolgus macaques, anti-AAV5 and anti-SaCas9 Abs were detected in 5 and 10 out of 20 animals, respectively. Subretinal injection of the AAV5 vectors induced robust Ab response to AAV5 but marginal, if any, response to SaCas9. More importantly, the presence of pre-existing or induced immunity to AAV5 or/and SaCas9 did not prevent the efficient AAV5 transduction of photoreceptor cells as demonstrated by in-situ hybridization of AAV vector genome in the outer nuclear layer, and the intracellular Cas9 expression shown by immunohistochemistry of Cas9 protein, as well as the targeted editing of CEP290 gene measured by deep sequencing in animals sacrificed at week 6 or 13 post dosing. In summary, we have demonstrated, using intended clinical device, robust delivery efficiency, target tissue specificity, and clinically meaningful activity of EDIT-101 and the surrogate NHP vector in non-human primates. In addition to the promising in vivo tolerability, further studies will be conducted to demonstrate adequate molecular specificity and safety profile to support EDIT-101 as a therapeutic candidate for the treatment of LCA10, which remains a highly unmet medical need.

681. Aberrant Clonal Hematopoiesis of the Erythroid and Myeloid Lineages in a Lentivirally Barcoded Rhesus Macaque

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Lentiviral vectors (LV) have been used for the delivery of genes into hematopoietic stem and progenitor cells (HSPC) in clinical trials worldwide. LV, in contrast to retroviral vectors, have not been associated with insertion site-associated malignant clonal expansions, and thus have been considered safer. Here, however, we present a case of aberrant, markedly dysplastic clonal hematopoiesis impacting the erythroid, myeloid and megakaryocytic lineages in a rhesus macaque transplanted with LV-barcoded HSPC. Autologous CD34+ HSPC from macaque ZL34 were transduced with an LV containing an MSCV promoter, GFP, and a high diversity barcode library, and reinfused following TBI. Transduction parameters and cell dose were similar to those of 14 other macaques transplanted with HSPC containing barcoded LV vectors manifesting highly polyclonal (thousands clones/animal) and stable hematopoiesis post-transplant. ZL34 initially engrafted with normal kinetics and blood count recovery. However, beginning 4-5 months (m) post-transplant, we observed marked eosinophilia and monocytosis, neutrophil dysplasia, and severe thrombocytopenia. Most strikingly, we observed elevated numbers of nucleated red blood cells (nRBCs) in the periphery, persisting at levels of up to 20,000/uL through euthanasia at 18.5m post-transplant. Bone marrow biopsy showed hypercellularity, marked dysplasia,

micromegakaryoctyes, erythroid predominance, and left-shift in all lineages but no increased blasts. The spleen contained extensive extramedullary hematopoiesis. These findings were classified as an overlap myeloproliferative/myelodysplastic disorder. The granulocyte, monocyte and nRBC lineages became virtually 100% GFP+ as the disorder worsened, identifying transduced HSPCs as the source of the disorder. High-throughput barcode and insertion site retrieval revealed complete dominance of a single clone with 9 LV insertions in the myeloid and nRBC lineages coincident with the clinical abnormalities. RNA-seq of nRBCs identified 3 genes, NCAM2, PLAG1, and KITLG, within 500 kB of an insertion, to be differentially upregulated. PLAG1 has been linked to murine AML in insertional mutagenesis studies. RNA-seq revealed upregulation of PLAG1 targets, such as IGF2, and aberrant splicing of PLAG1. Notably, PLAG1 is a gene downstream of HMGA2, of which upregulation has shown to associate with clonal expansion in a previous lentiviral HSPC gene therapy trial. These results suggest that the PLAG1 insertion may be fully or partially responsible for ZL34's MPD/MDS phenotype, although roles for dysregulation of NCAM2, KITLG or genes impacted by other insertions is possible. This case represents the first clear link between a lentiviral insertion-induced clonal expansion and a clinically abnormal transformed phenotype following transduction of normal primate or human HSPC, and are thus concerning, and suggest that strong constitutive promoters should not be included within LV vectors, and that targeted gene correction may be preferable to pursue for HSPC gene therapies.

682. Long-Term Efficacy and Safety Evaluation of the Administration of AAV9-Sulfamidase to the CSF of Dogs: 5-Year-Follow Up

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Mucopolysaccharidosis Type IIIA (MPSIIIA) is a rare autosomic recessive Lysosomal Storage Disease caused by deficiency in sulfamidase, a sulfatase involved in heparan sulfate (HS) degradation. Undegraded HS accumulates in lysosomes, resulting in severe progressive neurodegeneration with relatively mild somatic pathology. Patients usually die within the first 2 decades of life. We previously demonstrated in MPSIIIA mice that the intra-cerebrospinal fluid (intra-CSF) administration of AAV9 vectors encoding sulfamidase could mediate whole-body disease correction through transgene expression throughout the CNS and liver. Here, we evaluated the long-term therapeutic efficacy and safety of this gene therapy approach in a large animal model. Healthy Beagle dogs received an AAV9 vector encoding canine sulfamidase (AAV9-cSulfamidase). Transgene expression was followed for >4 years through measurement of sulfamidase activity in CSF. Safety was evaluated through biochemical and hematological parameters, magnetic resonance imaging of the brain and spinal cord, and abdominal ultrasounds. A full neurological evaluation was also performed. Liver biopsies were obtained to study persistence of vector and transgene expression in the liver of injected animals. A single, intra-CSF AAV9-cSulfamidase administration to dogs, at a clinically relevant dose, resulted in a long-term, stable increase in sulfamidase activity in CSF, in the absence of any safety concerns. Altogether these results demonstrate the long-term efficacy and safety of intra-CSF AAV9 sulfamidase gene transfer and support the clinical translation of this therapeutic approach for the treatment of MPSIIIA and other LSD with CNS involvement.

683. AAV-Associated Axonopathy of the Spinal Cord and Peripheral Nerves in Laboratory Animals: a Retrospective and Prospective Study

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Adeno-associated viral (AAV) vectors are increasingly at the forefront of treatment and research of genetic diseases, especially in regard to neurologic disorders. As more safety and tolerability studies are performed using AAV9 and AAV9-related vectors that target the central nervous system (CNS), a subtle and, in certain circumstances not so subtle, histologic finding within the spinal cord and peripheral nerves has emerged. Referred to as an axonopathy, the finding consisted of axonal degeneration of the dorsal white matter tracts of the spinal cord and peripheral nerves. Following AAV gene transfer the dorsal root ganglia (DRG), that project in the dorsal white matter tracts of the spinal cord and peripheral nerves, exhibited neuronal cell body degeneration, which was often observed in conjunction with a mononuclear cell infiltrate. The dorsal root ganglion is composed of sensory (afferent), pseudounipolar neuronal cell bodies and are involved in nociception and mechanotransduction. High level transduction of DRGs occurs via the circulation since they reside outside the CNS and have porous capillaries and via intrathecal administration by targeting the cell bodies or the axons. These findings have been observed to varying degrees in nonhuman primates (NHP) with multiple routes of administration including lumbar puncture (LP), intra-cisterna magna (ICM), and intravenous (IV), as well as in piglets administered vector IV. While the etiology is unknown, possible explanations include direct toxic effects of high neuronal transduction in the DRG and/or host immune responses to the transgene. Factors affecting the severity of these lesions could include species, age, route of administration and/or dose. A few reports in the literature suggest that reduced neuronal activity and cell death associated with AAV-mediated transduction in the dopaminergic neurons of the substantia nigra in rats may be caused by cell stress induced by both protein and RNA overexpression. In some cases, this has even been used to model certain neurodegenerative diseases, such as Parkinson's disease. However, the host immune response to a non-self-transgene may also play a role. The significance of the DRG, spinal cord, and peripheral nerve findings is not currently known as associated clinical signs have

thus far only been documented in piglets administered high dose AAV intravenously. Regardless, the possible impact of these findings on human clinical trials utilizing CNS-targeted AAV therapy supports the need for further investigation.

684. pH-Sensitive Multifunctional Lipid ECO Plasmid DNA Nanoparticles as Efficient Non-Viral Gene Therapy for Stargardt's Disease

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Monogenic retinal dystrophies can be treated with gene replacement therapy (GRT) due to easy localized delivery into retina and its immune privilege. Although adeno-associated virus vectors (AAV)-mediated gene therapy has recently become available for clinical use, AAVs have a limited loading capacity of 4.7 kb, restricting their applications in therapies for diseases that require delivery of large genes, such as Stargardt's disease (STGD). Previously, we developed a pH-sensitive multifunctional lipid ECO gene delivery system, which is not limited in cargo capacity, low cost, easy to prepare, and has shown gene transduction in the retina. In this study, we established and evaluated a nanoparticle-based GRT for STGD using ECO and tissue-specific therapeutic ABCA4 plasmid. Therapeutic plasmids expressing ABCA4 under control of a rod photoreceptor-specific RHO promoter and non-specific CMV promoter were designed and cloned. ECO and ABCA4 therapeutic plasmids were self-assembled to uniformed-size nanoparticles at an N/P ratio of 10 and subretinally injected into the *Abca4*$^{-/-}$ mouse model of STGD. ECO facilitated pH-sensitive amphiphilic endosomal escape and reductive cytosolic release (PERC) of the therapeutic plasmids in the retinal tissue. Seven days after injection, ABCA4 expression in the retinal tissue was evaluated using IHC and qRT-PCR. ABCA4 expression was observed in treated eyes for both tissue-specific (RHO) and non-specific (CMV) plasmids, which demonstrated significantly higher expression at the protein and RNA levels compared with sham injections. Furthermore, targeted ECO/pRHO-ABCA4 nanoparticles generated photoreceptor specific expression compared with ECO/pCMV-ABCA4 nanoparticles shown by both IHC and PCR. To evaluate the functional efficacy of GRT, the levels of A2E in the eyecup (pathological marker) were analyzed by HPLC 6 months after treatment. The treatments by both particles demonstrated a significant decrease in A2E levels compared with sham, indicating the slowing down of STGD progression. Successful GRT of STGD using nanoparticles formulated by ECO and therapeutic ABCA4 plasmids was achieved as demonstrated by tissue specific expression of ABCA4 and slowing down of A2E accumulation and STGD progression up to six months. The non-viral ECO/therapeutic gene formulations are a promising GRT strategy for a broad range of visual dystrophies.

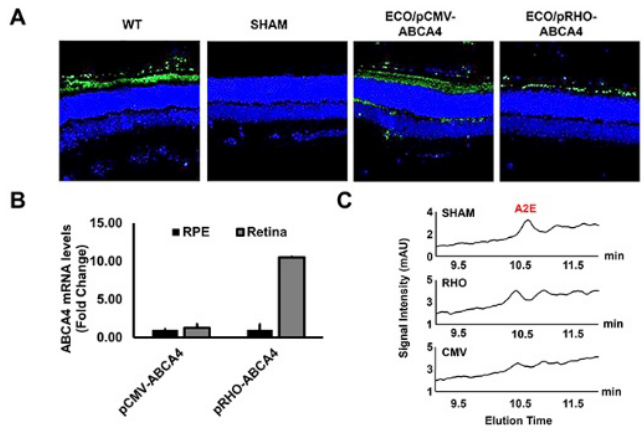

Figure 1. ABCA4 expression at protein and mRNA levels (normalized to RPE) in *Abca4*$^{-/-}$ mice after GRT demonstrated by **A)** IHC and **B)** qRT-PCR, respectively; **C)** A2E levels in *Abca4*$^{-/-}$ mice 6 months after GRT with non-targeted ECO/pCMV-ABCA4 and targeted ECO/pRHO-ABCA4 nanoparticles analyzed by HPLC.

685. Assessments of Liver-Targeted Hydrodynamic Gene Delivery in Hemophiliac Dogs

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Image-guided, liver-targeted hydrodynamic gene delivery was evaluated in hemophiliac dogs to assess its therapeutic effect in delivering factor IX gene into the hepatocyte. The electric power-driven injector was utilized in this study to test its safety and clinical applicability. Optimal parameters for the procedure were established using reporter construct (pCMV-Luc) in healthy dogs, including flow rate, injection volume, and intravascular peak pressure. The optimized parameters (flow rate: 20 ml/sec; injection volume: 1.3 times liver lobe volumes; intravascular peak pressure: 200 mmHg) were employed to assess and characterize the expression of human factor IX gene in 3 normal dogs (female, ~20 kg) using pBS-hFIX plasmids (100 μg/ml). The plasma concentration of human factor IX in treated dogs reached 22±2.1 μg/ml, 7 days after gene transfer, compared to 0.01 μg/ml in reporter plasmid-injected control, representing 37±7% of normal activity determined by coagulation assay using plasma from hemophilia B patients. The hFIX levels in treated dogs decreased with time and returned to background levels in 30 days. The same procedure was also performed in 2 hemophilia B dogs, replacing the human FIX gene with the canine FIX gene. The estimated cFIX level in treated dogs increased from background, undetectable levels to 19.6 and 6.5 μg/ml in 21 days after gene transfer, which gradually decreased with time. Other than a transient increase of transaminases, which showed peak level of approximately 250 IU/L which is 10 times higher than the normal level, on day 1 and recovered within a week, no other adverse events were observed in these dogs. Although additional work is needed, these results demonstrate the feasibility of using the procedure of liver-targeted hydrodynamic gene delivery for treatment of hemophilia.

686. A Minimally Invasive Procedure for Ultrasound-Mediated Nonviral Gene Delivery to Liver in a Porcine Model

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We achieved significant gene transfer using targeted, ultrasound (US)-mediated gene delivery (UMGD) of nonviral vectors in large animal models via a laparotomic procedure. The goal of this study was to develop a minimally invasive treatment protocol that involves utilization of transcutaneous, therapeutic US (tUS) to enhance gene transfer for ease of clinical translation. Because gene transfer efficiency was significantly reduced with transcutaneous UMGD due to US power attenuation across multiple tissue layers, we optimized the US transducers and protocols to overcome power loss while maintaining efficient gene delivery. Briefly, a balloon catheter was inserted into the pig hepatic veins of the target liver lobes via jugular vein access under the guidance of fluoroscopy. The balloon was then inflated to occlude blood flow. Ultrasound energy via the tUS transducer was localized by phospholipid microbubble (MB)-enhanced US imaging and delivered to the target liver lobe. tUS exposure was continuously applied to the lobe with simultaneous infusion of pGL4 plasmid (encoding a luciferase reporter gene) and MBs. tUS was delivered via an unfocused disc transducer (H105), a focused 4-element array transducer (H114) or a transducer (XDR106) with either an unfocused 5-element array or a focused 10-element array. Twenty-four hours after treatment, the liver lobes were harvested and spatially-mapped, and the gene expression was quantified. Analysis showed significant gene transfer in treated lobes using H105 and H114 in comparison to control lobes (without US exposure). Transcutaneous UMGD via H105 yielded a 100-fold increase of luciferase expression in the treated lobe compared to the control and obtained wide spatial distribution of gene transfer due to high treatment volume. H114 resulted in a 3000-fold increase of luciferase expression compared to control, with enhanced gene expression levels comparable to those achieved in pig livers by a laparotomy approach (>104 RLU/mg protein). To achieve therapeutic levels of gene expression by UMGD requires large treatment volume and high peak negative pressure (PNP) output, but a tradeoff needs to be adopted between these two factors. Therefore, XDR106 was designed with a novel array to overcome the barrier allowing an enlarged treatment volume without sacrificing PNP output and offering an optimal UMGD efficiency. Additionally, the increased range of applied PNP values (3.0 - 12.0 MPa) and gene expression induced minimal liver injury with alanine and aspartate transaminase values remaining within normal ranges. In conclusion, we successfully developed a technique for minimally invasive, transcutaneous US-enhanced gene transfer in pig livers with the guidance of fluoroscopy. We designed and optimized the utilization of novel US transducers to minimize power attenuation across several tissue barriers for efficient UMGD, which facilitates the clinical translation of this technique for treating patients with hemophilia.

AAV Vectors III

687. A Novel Genomics-Based Platform for the Creation of Environmental-Responsive Gene Promoters

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Recent advances in understanding how the human genome is regulated has afforded the opportunity to mine our DNA for novel regulatory elements that can drive exogenously delivered therapeutic genes in a much more specific and efficient manner. We have developed a gene promoter design and construction platform that integrates data derived from large-scale functional genomics datasets and employs machine learning algorithms in data analysis with a view to identify functional gene regulatory elements. Elements are then ranked according to many distinct criteria and subsequently used as component parts in synthetic promoter construction, using engineering biology principles. Here we introduce the platform and present results detailing how we have employed it to create cell type selective promoters in muscle, liver and retina target cell populations. We have shown in the liver that the activity of our synthetic promoter candidates is significantly higher than currently used industry standard liver-selective promoters, with activities far more than what is normally seen with constitutively active promoters, such as CBA/CAG. In muscle, we show that promoters are selectively active in mature differentiated myotubes and we are further able to show a large dynamic range of activity. Similarly, we show the activity of the synthetic RPE-selective promoter candidates are several-fold higher than what is achievable with endogenous RPE-specific promoters such as RPE65, CRALBP and Bestrophin. We have also used the platform to generate novel inducible promoters responsive to different chemical and biological stimuli, and whose activity is stimulated solely with the addition of an inducer without the requirement for the co-expression of a trans-activator. We present data showing their activities, highlighting the tightness of gene regulation that they mediate and showing how elements from different systems can be combined to create logic gates based on transcriptional regulation. In summary, we have developed a novel genomics-based platform that enables the rational design, synthesis and testing of mammalian gene regulatory elements, and whose output can help support the construction of the next generation of cell and gene therapeutics.

688. Enhancement of AAV Transduction Using Anti-Malarial Drugs, Hydroxychloroquine and Chloroquine

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The therapeutic effects of gene therapy using adeno-associated viral (AAV) vectors are dependent on the efficacy of viral transduction. In recessive retinal dystrophies, for instance, a transduction rate approaching 50% of photoreceptors or retinal pigment epithelial (RPE) cells is desirable to slow down or stop the retinal degeneration, since carriers are usually minimally affected. Increased vector dose must also be balanced against the need to avoid triggering deleterious inflammatory or immune responses. Significant improvements in the efficacy of AAV vectors have been achieved through modifications of the viral capsid, however little is known about innate anti-viral responses within the target cells which may restrict viral transduction. Using RT-qPCR, we detected upregulation of intracellular viral sensors (cGAS, STING, RIG-I, TLR9, TRIM21) and effectors (TNFα, CXCL10, ISG15, IFNγ, APOBEC3) in the mouse retina following subretinal gene therapy with an AAV2 vector encoding green fluorescent protein (GFP), but not in the sham-injected eyes. We postulate that suppression of intracellular immunity using the putative inhibitors, hydroxychloroquine (HCQ) and chloroquine (CQ) (both anti-malarials used in the treatment of lupus and rheumatoid arthritis) might enhance AAV transduction. Mouse embryonic fibroblasts (MEFs), non-human primate (NHP) RPE cells, and human retinal explants were treated with HCQ in the culture media for one hour prior to transduction with AAV2.GFP. GFP expression was quantified by flow cytometry in the MEFs on day 3, by RT-qPCR and western blot in NHP RPE cells on day 3, and by mean fluorescence grey value in human retinal explants on day 11. Compared with vector alone, adjunctive HCQ increased GFP expression by up to 3.4-fold in MEFs (1-way ANOVA: n=4, p=0.001), 3-fold by qPCR (n=3, p=0.025) or 4-fold by western blot (n=2) in NHP RPE cells, and 2-fold in human retinal explants (n=6, p=0.021). Positive dose response of AAV transduction was seen in MEFs and NHP RPE cells to HCQ up to 18.8 μM. Similar dose response was also seen in MEFs to CQ up to 12.50 μM (associated with 3-fold increase in GFP expression). To test the safety and efficacy of HCQ in vivo, subretinal injections of AAV2.GFP with or without 18.8 μM HCQ were performed in matched eyes of 7-week old C57BL/6J mice. In vivo scanning laser ophthalmoscopy showed that mean retinal fluorescence (486 nm, representing GFP expression) was 2-fold higher in eyes that received gene therapy with HCQ at 4 and 8 weeks, relative to those that received vector only (2-way ANOVA: n=5, p=0.006). Optical coherence tomography showed no difference in retinal thickness (n=5, p=0.431) or morphology between eyes treated with or without HCQ, indicating absence of retinal toxicity. The results suggest that a single pulse of adjunctive HCQ, CQ or related compounds could enhance AAV transduction in multiple cell types and across species. Subretinal gene therapy with HCQ appeared to be safe and led to sustained increase in transgene expression.

689. Genetic Modification of the AAV5 Capsid Resulting in De-Targeted Liver and Enhanced Vector Delivery to Lung Following Intravenous Administration

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Intravenous administration of all naturally occurring adeno-associated virus (AAV) vectors are liver tropic, with the majority of the total vector dose mediating gene expression in liver hepatocytes. To design an AAV vector to de-target liver but enhance delivery to lung following intravenous administration, we hypothesized that adding additional positively charged lysine residues to the AAV capsid would enhance the ability of the virus to adhere to negatively charged cell surface molecules such as heparan sulfate proteoglycans, leading to enhanced transduction of the organ representing the first capillary bed encountered. To test this hypothesis, using site directed mutagenesis, two lysine residues were inserted into variable loop VIII of the AAV serotype 5 capsid of a vector coding for human α1-antitrypsin (AAV5-PK2-hAAT). Distribution of the vector and vector expression was quantified following administration of 10^{11} genome copies (gc) of AAV5-PK2-hAAT or the identical vector with the AAV5 wild-type capsid (AAV5-hAAT) by the intravenous route to C57Bl/6 male mice. Four wk post-administration, organs were harvested, and vector DNA and mRNA quantified by TaqMan. Consistent with the experience for all naturally occurring AAV vectors, intravenous administration of AAV5-hAAT resulted in the highest vector DNA levels in the liver (ratio of vector DNA in liver to lung 150 ± 71; liver to heart 452 ± 169; and liver to skeletal muscle 4260 ± 1800). hAAT mRNA levels in the various organs paralleled the vector DNA distribution. In striking contrast, following intravenous administration of the AAV5-PK2-hAAT vector, the liver to lung vector DNA ratio was 0.17 ± 0.06 (p=0.03, compared to the wild type capsid), liver to heart 84 ± 48, (p=0.03) and liver to skeletal muscle 671 ± 319, p=(0.04). The lysine-modified vector was de-targeted from the liver, resulting in 61-fold lower (p=0.003) liver and 2.3-fold higher (p=0.03) lung vector DNA levels compared to the wild-type capsid (lung to liver DNA ratio 276-fold higher, p=0.04). The hAAT mRNA levels paralleled the vector DNA levels. Serum AAT levels were 84 ± 20 μg/ml for AAV5-hAAT and only 0.57 ± 0.15 μg/ml for AAV5-PK2-hAAT supporting the de-targeting of AAV5-PK2-hAAT to the liver, the main producer of secreted proteins such as hAAT. In summary, genetic modification of the AAV capsid with lysine residues results in a "sticky" vector that transduces the organ containing the first capillary bed encountered following intravenous administration (the lung), thus shifting the targeting of the vector away from the liver. This capsid modification strategy with lysine residues should be applicable to all AAV vectors, and depending on the administration route, useful for targeting AAV vectors to specific organs, and also useful from a safety viewpoint for limiting "spill" of vector to the systemic circulation and other organs.

690. Evaluating Safety and Potency of the AAV2tYF-GRK1-RPGRco Vector in RPGR-Deficient Rd9 Mice

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Purpose: X-linked retinitis pigmentosa (XLRP) accounts for approximately 10% of all RP cases, and approximately 80% of XLRP cases are caused by mutations in the retinitis pigmentosa GTPase regulator (*RPGR*) gene. AGTC is developing a recombinant adeno-associated virus (rAAV) vector AGTC-501, also designated AAV2tYF-GRK1-RPGRco, to treat RP patients with mutations in *RPGR*. The vector contains a codon-optimized human *RPGR* cDNA (RPGRco) driven by a photoreceptor-specific promoter (G protein-coupled receptor kinase 1, GRK1) and is packaged in an AAV2 capsid with three surface tyrosine residues changed to phenylalanine (AAV2tYF). Here we report results of a GLP safety and potency study of this vector administered by subretinal injection in the naturally occurring RPGR-deficient Rd9 mouse model. **Methods:** AAV2tYF-GRK1-RPGRco was manufactured using AGTC's proprietary herpes simplex virus (HSV)-based method. Sixty Rd9 mice (20 per group; 6-8 weeks of age) received a 1µL subretinal injection in the right eye of vehicle (control) or AAV2tYF-GRK1-RPGRco at one of two dose levels (4×10^8 or 4×10^9 vg/eye). The left eye from all animals was untreated. Ten animals per group were sacrificed at 4 weeks and the remaining animals were sacrificed 12 weeks after injection. Toxicity assessment was based on mortality, clinical signs, body weight, ophthalmic exams, scotopic and photopic ERGs, organ weight, and clinical and anatomic pathology. Potency was assessed by immunohistochemistry (IHC) of RPGR expression in the retina. **Results:** No vector-related morbidity, mortality, clinical signs, or changes in body weight were observed. No vector-related changes in organ weight, macroscopic findings, or hematology/clinical chemistry parameters were present. There were no vector-related changes in ophthalmic exams or scotopic/photopic ERG responses. Microscopic findings were characterized by the presence of pigmented cells in the subretinal space and within the photoreceptor cell layer, and degeneration of photoreceptor and thinning of inner and/or outer nuclear layer in 55% of injected eyes. In addition, swollen lens fibers and lens fibrosis were present in 10% of injected eyes. Because these findings were dose-independent and present across all groups, including vehicle controls, they were considered procedure-related and not vector-related. Immunolabeling of RPGR protein, mainly in the inner segment of photoreceptors and the adjacent connecting cilia region, was observed in all the vector-treated eyes examined at both dose levels and the expression level was in a dose-dependent manner. No serum antibody to human RPGR protein was detected in any animals at any time point. Due to the slow progression of retinal degeneration in Rd9 mice, a therapeutic effect on ERG responses was not expected and was not observed in this 3-month study.**Conclusions:** Subretinal injection of AAV2tYF-GRK1-RPGRco in RPGR-deficient Rd9 mice induced predominant RPGR expression in the retina and was well tolerated with no vector-related effects at either 4×10^8 or 4×10^9 vg/eye. The no observed adverse effect level (NOAEL) for this study was considered to be 4×10^9 vg/eye.

691. Mapping the Connectome Using Novel AAV Vectors, DNA Barcoding and Spatial Transcriptomics

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Recent advances in sequencing has opened up for spatially resolved RNA identification. One such technology, utilized here, is Spatial Transcriptomics (ST). Using novel barcoded AAV vectors with efficient retrograde transport to neurons, followed by ST mapping, we here present a more precise method to evaluate vector transport, vector function and mapping connectivity in the brain. This approach enables the mapping of molecular barcodes to defined brain regions with simultaneous RNAseq mapping of cellular phenotype. As such, this method provides significant advancement over current state-of-the-art methods. We believe that this method will be highly useful in studies regarding viral vector screening and it could also be used for mapping the connectome. We also utilized this application for looking at transplanted cells in the brain. Brain repair using embryonic stem cell (ESC) transplantation in Parkinson's disease has shown great promise as a future treatment option. However, little is known what directs maturation and circuit integration. In this study, we transplanted human ESC-derived dopaminergic neurons into a 6-OHDA lesioned rat model and utilized retrograde infectivity of novel AAV's in combination with mono-synaptic rabies tracing, molecular barcoding and spatial transcriptomics to answer these questions.

692. Massively Parallel *In Vivo* Characterization of >150 Adeno-Associated Viral (AAV) Capsids Using DNA/RNA Barcoding and Next-Generation Sequencing

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Over the last decade, the ever increasing repertoire of techniques for molecular evolution of AAV capsid proteins has resulted in a massive expansion of the available pool of synthetic viral particles, comprising chimeras, mutants or peptide-modified derivatives of natural AAV isolates. Concurrently, this has intensified the need for novel experimental schemes that permit to rapidly screen and stratify this wealth of AAV capsids in a high-throughput manner, ideally within the complex physiological environment of living animals. To this end,

we adapted a previously reported strategy in which a given AAV capsid variant is loaded with a viral genome that is tagged with a specific DNA barcode, permitting its qualitative and quantitative tracking *in vivo*. Here, we improved this design by placing the barcode into the 3'UTR of an AAV vector genome expressing a YFP reporter from a CMV promoter, thus ensuring that the barcode becomes part of the vector-encoded transcript and hence enabling simultaneous capsid tracking on the DNA and RNA level. This is key as it facilitates the identification of ideal capsids for gene therapy applications, delivering little to no vector DNA in unwanted off-target tissues, while mediating strong and specific transgene expression in the desired on-target. Using this design, we produced 3 barcoded AAV libraries comprising >150 capsid variants, including 12 commonly used wild-types, >70 novel peptide display mutants based on these natural AAVs, as well as an array of published benchmarks such as AAV-DJ, -LK03, -Anc80L65 and -PHP.B. Following i.v. delivery of these libraries to female or male adult C57BL/6 mice, we collected >20 tissues and then assessed the biodistribution (DNA) and activity (RNA) of all capsids via next-generation sequencing and qPCR. Notably, our data confirm and expand prior observations for the benchmark capsids, such as a robust liver tropism of AAV-DJ or a pronounced activity of AAV-PHP.B in the brain, inherently validating our work-flow. Moreover, for other capsids that were reported to mediate strong expression in selected tissues, we detected substantial amounts of transcriptionally inactive DNA in off-target organs, thus raising concerns about the use of these variants in gene therapy. Most remarkably, we identified a peptide that radically changes the *in vivo* biodistribution of AAV9 by re-targeting >70% of the resulting capsid, termed AAVMYO, to muscle tissues. Compared to AAV9, AAVMYO mediated 10.6-fold (diaphragm), 7.2-fold (quadriceps) and 1.6-fold (heart) higher transgene expression in the context of the barcoded library. Separate validation of this novel capsid verified its unique efficiency and specificity, with 11- to 55-fold higher gene expression than AAV9 in muscle tissues, concurrent with a 9-fold detargeting in the liver as the major off-target. In conclusion, we illustrate the enormous potential of combinatorial DNA/RNA barcoding of AAV capsid libraries, best exemplified by our discovery of a promising new candidate for muscle-directed gene therapies.

693. A Simple Strategy for Augmenting the Transduction Efficiency of AAV Vectors: Role of Essential Metal Ions

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Metal elements are essential components of approximately half of all cellular proteins, and approximately one-third of all known enzymes thus far are metallo-enzymes. Since several cellular proteins and enzymes impact the transduction efficiency of recombinant AAV vectors, the precise role of metal ions in this process has not been studied extensively. For example, there are only two published reports that have documented that the transduction efficiency of AAV vectors can be enhanced by arsenic trioxide (Mitchell *et al*., *J. Virol*., 87: 4571-4583, 2013) and sodium chloride (Adamson-Small *et al*., *Hum. Gene Ther. Meth*., 28: 1-14, 2017), respectively. We have initiated a systematic study in which we wish to evaluate the role of all essential metal ions on the transduction efficiency of AAV vectors. In the initial set of experiments, HeLa cells were transduced in triplicates with scAAV2-EGFP vectors at an MOI of 200 vgs/cell, followed by addition of various concentrations of magnesium chloride or zinc chloride. Transgene expression was determined 48 hrs post-transduction using fluorescent microscopy, and images were analyzed using the ImageJ software. The results showed a dose-dependent increase in transgene expression, with maximal increase with 100 υg/ml of magnesium chloride (~2-fold), and with 30 υg/ml of zinc chloride (~8-fold). Interestingly, when cells were treated with a combination of both magnesium chloride and zinc chloride, the increase in the transduction efficiency was more than additive (~14-fold). These results are shown in Figure 1. Studies are currently underway to evaluate the role of all essential metal ions, either alone, or in various permutations and combinations, to achieve the optimal enhancement in AAV vector-mediated transgene expression, both *in vitro* and in a murine model *in vivo*. Our results suggest that this simple strategy of essential metal ions-mediated enhancement may be applicable to other AAV serotype vectors, which has important implications in the optimal use of AAV vectors in human gene therapy.

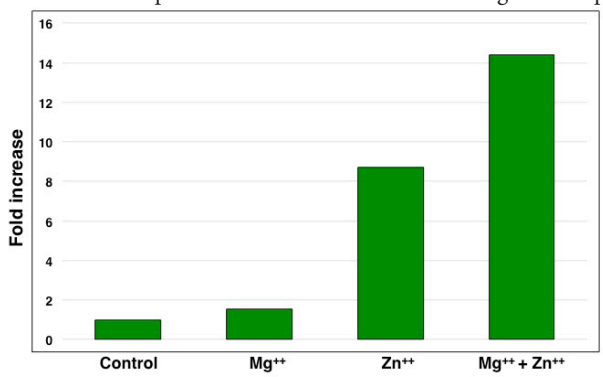

Figure 1: Effect of Mg^{++}, Zn^{++}, and Mg^{++} + Zn^{++} on the transduction efficiency scAAV2-EGFP vectors.

694. Separation, Interchange and Chimerization of the N- and C-Terminal Halves of the AAV AAP Proteins Reveals Their Functional Domains

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Adeno-associated virus (AAV) assembly activating proteins (AAPs) have been shown to play an essential role in capsid assembly. However, recent studies have demonstrated that the AAP protein is not an absolute requirement for capsid assembly for AAV4, 5, and 11. In addition, assembly of AAV9, and several other AAP-dependent serotypes, takes place with a mutant AAP devoid of the C-terminal half of the protein. Moreover, serotype cognateness between VP and AAP proteins is not crucial for AAP-dependent capsid assembly, with the rare exception of Snake AAV. Thus, despite the unambiguous role of AAP in capsid assembly, basic AAP biology and capsid assembly remain an unsolved puzzle. Here, we take a reptile AAV-based approach

to decipher this puzzle and show distinct modular roles of the N- and C-terminal halves of AAP (AAPN and AAPC, respectively). Our approach takes advantage of the discovery that Snake AAV requires strict serotype cognateness between the VP and AAP proteins for capsid assembly while AAV2, 8, 9, and Dragon AAV are promiscuous with regards to viable VP-AAP complementation. In this study, we constructed plasmids expressing the wild-type AAPs (AAPNCs), AAPNs, AAPCs, AAPCNs (NC-to-CN-flipped AAPs in which AAPN and AAPC are interchanged and the original N- and C-termini are reconnected by a short linker), and AAPNC chimeras composed of heterologous AAPNs and AAPCs. The wild type and these AAP mutants were then studied in HEK293 cells using a VP-AAP trans-complementation assay for VP3 capsid assembly, immunofluorescence microscopy, VP-AAP co-immunoprecipitation, and western blotting (WB). AAPCs attained higher steady-state expression levels compared to the wild type but were incapable of promoting capsid assembly. AAPNs were undetectable by WB but were capable of supporting assembly of AAV8, AAV9, and Snake AAV capsids. Interestingly, AAPC-independent assembly of Snake AAV did not occur with its cognate AAPN Snake, but took place only with AAPN Dragon, indicating that AAPC-independency is context-dependent and is not necessarily determined solely by the nature of VPs. Co-expression of AAPN and AAPC did not complement functions missing in each truncated mutant, but covalent linkage of AAPC and AAPN in a flipped orientation (i.e., AAPCN) restored full AAP functions. Serotype specificity and promiscuity of AAPNC chimeras were determined by the serotype from which the AAPN was derived. Binding of VP to a functionally-competent AAP was not necessarily sufficient to support capsid assembly, indicating a post-binding mechanism to determine serotype specificity. These observations demonstrate that the functional domains of AAPs are in a modular organization such that AAPN determines serotype specificity for assembly and AAPC stabilizes the protein. Thus, reptile AAVs provide an insightful opportunity to study the puzzle of AAP biology.

695. Developing AAV Vectors for More Efficient and Selective Gene Expression in Specific Cell Types of the Nervous System Following Systemic Delivery

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Recombinant adeno-associated viruses (rAAVs) are commonly used as gene delivery vehicles in biomedical research and have shown great potential for gene therapy. Systemic administration of AAVs can be used to achieve broad vector distribution. However, key challenges remain with systemic administration of AAVs as their lack of organ and/or cell-type specificity may cofound experiments and cause off-target effects. We and others have evolved AAVs to direct their tropism towards specific organs, such as the brain, after systemic delivery. We previously described CREATE, a Cre-based selection method, and used it to develop AAVs that efficiently transduce the central nervous system (CNS) in adult rodents, namely AAV-PHP.B (Deverman et al, Nat. Biotech., 2016), a further enhanced variant, AAV-PHP.eB (Chan et al., Nat. Neurosci., 2017), and AAV-PHP.S (Chan et al., Nat. Neurosci., 2017), a variant that can efficiently transduce the peripheral nervous system (PNS) and several visceral organs. While exhibiting improved efficiency, these capsids are not specific to their targets. To identify variants that target a cell-type or organ of interest more specifically, we incorporate both positive and negative selection into the CREATE method. We aim to achieve this by performing *in vivo* parallel selections across multiple mouse Cre transgenic lines coupled with next-generation sequencing of libraries at each step (CREATE2.0). This approach provides enrichment scores of each variant in the library across selections, enabling the *in silico* discovery of variants that are selectively enriched in target cell-types, but not in other off-target cell-types or organs. Using this method, we have identified AAV-PHP.B2N, a capsid that preferentially targets CNS neurons and has reduced liver transduction, and AAV-PHP.B2A, a capsid that is biased towards astrocytes. In parallel to these capsid engineering efforts, we have been designing and validating gene regulatory elements for transcriptional and post-transcriptional control of transgene expression to limit expression to specific neural cell-types, including neurons, astrocytes and oligodendrocytes after systemic AAV delivery. We have developed vectors with transgenes controlled by regulatory elements from the human HTT and FXN genes that may have applications for therapeutic gene transfer, in Huntington's disease and Friedreich's ataxia, respectively. Collectively, the versatility and efficiency of the new CREATE method, novel viral capsids, and regulatory elements can expand the available options for achieving targeted gene expression in specific CNS cell populations via non-invasive, systemic delivery.

696. Evaluating Effector Function as a Determinant of ADA against AAV-Delivered Antibodies

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Human vaccine trials suggest that antibody Fc-domain effector functions contribute to protection from HIV-1 infection. However, we have shown that adeno-associated virus (AAV) expression of the antibody-like molecule, eCD4-Ig, with attenuated effector functions of rhesus IgG2, completely protected rhesus macaques from SHIV-AD8 challenges. In this study, we compared AAV expression of rhesus IgG1 and IgG2 forms of eCD4-Ig and of four broadly neutralizing antibodies (bNAbs) recognizing the CD4-binding site (3BNC117, NIH45-46) or the N332-glycan site (10-1074, PGT121) of the HIV-1 envelope glycoprotein (Env). All bNAbs elicited strong anti-drug antibody (ADA) responses in macaques, but IgG2-isotyped bNAbs exhibited significantly higher peak concentrations, lower ADA, and subsequently greater protection against SHIV-AD8 than did their IgG1 counterparts. Similarly, rh-eCD4-IgG2 expressed higher peak and post-peak concentrations and elicited fewer ADA than did rh-eCD4-IgG1. Our data indicate that the rhesus IgG1 Fc domain more consistently elicits ADA than does the IgG2 domain, and suggests that IgG2 might help limit ADA responses that interfere with the activities of other therapeutic antibodies and biologics. Surprisingly, in studies

characterizing the antibody-dependent cell-mediated cytotoxicity (ADCC) activity of eCD4-Ig and antibodies, the rhesus IgG2 forms of these molecules, as opposed to human IgG2, retain some ADCC activity. While this activity may be important for protection from HIV-1 infection, engagement of the immune system may be inducing higher ADA responses. We hypothesize that limiting the engagement of AAV-expressed antibodies with the Fc-gamma receptors (FCGR), may limit ADA against the expressed antibody. To examine this, we designed an effector-function-null Fc domain in the cynomologous macaque (*Macaca fascicularis*) background. This Fc domain includes the described LALA mutations and P329G to abrogate FCGR binding as well as two additional mutations (D270A, K320A) to further abrogate complement binding and fixation. Studies assessing the immunogenicity of these constructs when expressed from AAV in cynomologous macaques are on-going. Here we report *in vitro* characterization of these mutant antibodies for their binding to FCGR and ability to fix complement. Limiting ADA will increase the efficacy of AAV-delivered antibodies in future applications.

697. Enhanced Analgesic Effect of AAV-Encoded Mutant CBD3 Peptide (CBD3A6K) for Primary Sensory Neuron-Targeted Treatment of Established Neuropathic Pain in Rat

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N-type voltage-gated calcium channels ($Ca_V2.2$) of primary sensory neurons, whose cell bodies lie in the dorsal root ganglia (DRG) and trigeminal ganglia, mediate neurotransmission of pain in the dorsal horn of the spinal cord. The calcium channel-binding domain 3 (CBD3) peptide (15aa) derived from the collapsin response mediator protein 2 (CRMP2) attenuates pain by reducing inward Ca^{2+} currents through the pore-forming $Ca_V2.2\alpha1b$ subunit by blocking its binding to CRMP2. While effective in providing pain relief, the therapeutic potential of systemic application can be compromised by undesired off-site effects due to broad blockage of multifunctional $Ca_V2.2$. We previously showed that adeno-associated viral (AAV)-mediated restricted expression of CBD3 peptide in the peripheral sensory nervous system prior to a nerve injury prevents the development of pain, providing proof of the utility of this strategy for prophylactic uses. In this study, we tested the potential analgesic efficacy of DRG-targeted AAV-CBD3 in the treatment of established neuropathic pain. Lumbar (L) 4 and 5 intraganglionic delivery of AAV6-encoding recombinant fluorescent (EGFP) CBD3 (AAV6-CBD3), as well as the control vector of AAV6-EGFP, were performed in rats 2-week after induction of neuropathic pain by tibial nerve injury (TNI). Efficient transgene expression in DRG neurons and their axonal projections was observed 4 weeks after vector delivery, indicating that TNI did not affect AAV-mediated transgene expression. However, AAV6-CBD3 injection did not significantly reduce pain behaviors including mechanical and cold hypersensitivity, compared to TNI animals injected with the AAV6-EGFP control vector. We next constructed AAV6-EGFP-CBD3A6K (AAV6-CBD3A6K) expressing a fluorescent CBD3A6K (replacing A to K at position 6 of CBD3 peptide), which is conformationally more rigid than the parental CBD3, resulting in more potent and specific block of $Ca_V2.2$. Delivery of AAV6-CBD3A6K into L4/L5 DRGs initiated 2 weeks after TNI induced transgene expression in L4/L5 DRGs and their axonal projections, and produced significant attenuation of pain behavior in the TNI rats compared to controls (AAV6-EGFP injection). As a potential mechanism, we observed that the increased $Ca_V2.2\alpha1b$ immunoreactivity in the ipsilateral spinal dorsal horn of TNI animals was normalized by AAV6-CBD3A6K treatment. Collectively, these results indicate that DRG-restricted AAV6 delivery of CBD3A6K is a more effective analgesic molecular therapy than AAV6-CBD3 for the treatment of established neuropathic pain in rats, and is an instructive example of how pain therapeutics can be tailored to various pain subtypes.

698. Abstract Withdrawn

699. Improving AAV8 Infectivity by Swapping with AAV2 Phospholipase A2 Domain

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Adeno-associated virus serotype 8 (AAV8) is one of the most efficient vectors for transduction of mouse hepatocytes. However, AAV8 has low infectivity for *in vitro* cell cultures, which hinders its *in vitro* assays. Since AAV2 has the highest infectivity for *in vitro* cell cultures, we reasoned that replacing the phospholipase A2 domain of AAV8 with that of AAV2 might improve the infectivity of AAV8 in *in vitro* cell cultures. To test this hypothesis, we constructed a chimeric capsid gene by deleting the first 132-amino acid sequence from AAV8 VP1 and replacing it with the first 132-amino acid sequence from AAV2 VP1 and named it as Cap8.2. Recombinant baculovirus (rBV) carrying this chimeric capsid and AAV2 rep genes (rBV-Cap8.2-Rep) was generated. AAV8.2 vectors were produced in Sf9 cells through co-infection with rBV-Cap8.2-Rep and a second rBV carrying a reporter gene flanked by AAV2 ITRs. Results indicate that this chimeric modification did not negatively impact the production yield and exceeding 1e+15vg purified AAV8.2 vectors were obtained from each liter of Sf9 cell culture. Repeated experiment results show that AAV8.2 has 2~3 folds higher infectivity in *in vitro* transduction assays than AAV8, indicating that the phospholipase A2 domain from AAV2 indeed improved the *in vitro* infectivity of AAV8 vectors. Then we performed *in vivo* study with BALB/c nude mice to see if there was any difference in the infectivity between AAV8.2 and AAV8. Experimental results indicate that one week after tail vein injection, AAV8.2 showed slightly better luciferase expression than AAV8. Two weeks after tail vein injection, however, AAV8.2 expressed significantly higher luciferase level as compared with AAV8, indicating that the AAV2 phospholipase domain did improve the infectivity of AAV8 vectors *in vivo*. The luciferase expression level of AAV8.2 gradually decreased to similar level of AAV8 four weeks after tail injection. These data demonstrate that AAV2 phospholipase A2 domain indeed enables stronger infectivity of the AAV8.2 vectors produced in Sf9 cells both in vitro and in vivo.

700. Identification and Characterization of an Alternate, AAVR Independent, AAV Entry Mechanism Using a Genome-Wide CRISPR/Cas9 Knock-Out Screen

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Determinants and mechanisms of cell attachment and entry steer the Adeno-Associated Virus (AAV) in its utility as a gene therapy vector. Thus far a systematic assessment of how diverse AAV serotypes engage their proteinaceous receptor AAVR (KIAA0319L) to establish transduction has been lacking, despite potential implications for cell and tissue tropism. Here, a large set of human and simian AAVs as well as *in silico* reconstructed ancestral AAV capsids were interrogated for AAVR usage. We identified a distinct AAV capsid lineage comprised of AAV4 and AAVrh32.33 that can bind and transduce cells in the absence of AAVR, independent of multiplicity of infection. Serotypes independent of AAVR are able to transduce WT cells to an equal level as AAVR KO cells, and are unable to be rescued by AAVR re-introduction in a non-permissive cell line. Cell binding and viral overlay assays demonstrate that these serotypes are also unable to bind to the AAVR protein. Importantly, AAVrh32.33 is able to transduce *Aavr* KO and WT mice to a similar level, demonstrating that these AAVs use an alternate entry mechanism *in vivo*. We have additionally carried out a genome-wide CRISPR/Cas9-based knock-out screen to identify entry factors required for this alternate AAV entry pathway. Multiple rounds of selection followed by deep sequencing of cells refractory to AAV transduction have identified several important cellular factors involved in this alternate AAV entry pathway employed by AAV4 and AAVrh32.33. By elucidating serotype specific entry pathway differences we aim to further inform the development of AAV as a gene therapy vector.

701. Optimization of the Production of rAAV2/HBoV1 Vector

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Human bocavirus 1 (HBoV1) is a human respiratory virus that naturally infects the human respiratory track. Cross-genus pseudopackage of a recombinant AAV2 (rAAV2) genome into the HBoV1 capsids led to the development of the chimeric parvovirus vector rAAV2/HBoV1, which eliminates the concern about the HBoV1 pathogenicity and expands the package capacity of the rAAV genome by 20% (up to 5.9kb). rAAV2/HBoV1 provides a solution for the current lack of an efficient airway transduction vector that can package large transgenes such as CFTR for the treatment of cystic fibrosis. Unlike AAV, the expression plasmid encoding HBoV1 capsid protein open reading frame (*cap* ORF) or cDNA failed to express HBoV1 capsid proteins. In the prototype vector production system, the trans complementation of HBoV1 capsid is provided through a clone of HBoV1 replication incompetent genome (pHBoV1NSCap), which expresses all the nonstructural proteins (NS1-4 and NP1) and capsid proteins (VP1-3). To optimize the vector production, the involvement of each nonstructural protein was investigated. We found that elimination of the NS1 and NS2 expression significantly increased the vector production, and the expression of NS3 and NS4 are not essential. We also found that NP1 was the minimal nonstructural component required in complement recombinant rAAV2/HBoV1 vector production in trans. New helpers, pCMVm630NS1(-)NP1cap-AV2rep and pCMVm630NP1cap-AV2rep were constructed by either knocking out the NS1/2 expression from the pHBoV1NSCap through early termination of ORF or completely deleting the coding sequence of NS1-4. Additionally, the endogenous P5 promoter was replaced by CMV early enhancer/promoter to strengthen the HBoV1 gene transcription and the AAV2 *rep* expression cassette was incorporated to simplify the production from 4-plasmids co-transfection to 3-plamsids in HEK293 cells. With these helpers, the production yield of rAAV2/HBoV1 was increased to 4-7×10^3 DRP/cell, 8-fold higher than that using the prototype helper. Studies also revealed that the transcription of *cap* mRNA requires the function of NP1 to read through the proximal polyadenylation sites [(pA)p] at the VP1/2 unique region (VP1/2u) of the *cap* ORF. Codon optimization of the coding sequence of VP1/2u by silent mutations to deplete the (pA)p eliminates the requirement of NP1 for HBoV1 *cap* expression. NP-1 independent helper was developed using the VP1/2u-optimized *cap* ORF, which yielded rAAV2/HBoV1 at production efficiency and transduction potency similar to those produced from the helper pCMVm630NS1(-)NP1cap-AV2rep or pCMVm630NP1cap-AV2rep. These systems will improve the ability to preclinical test rAAV2/HBoV1 vectors in models such as the cystic fibrosis ferret.

702. Transduction Efficiency of Novel AAVs in Mammalian Inner Ear

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Background: Inner ear gene therapy is a promising treatment for hearing loss and dizziness. One of the major challenges of inner ear gene therapy is identifying a viral vector which can infect target cells with high specificity and efficiency. In this study, we examined several novel AAVs' ability to infect various cell types in the mouse inner ear. **Methods:** Neonatal (P0-P5) CBA/J mice were used in this study. Several AAV-GFPs (AAV2-7m8, AAV-DJ, and AAV8BP2) were injected into mouse inner ear using the posterior semicircular canal approach. Immunohistochemistry was used to assess the infection efficiency. ABR was used to assess auditory function. **Results:** Of the AAVs tested, AAV2-7m8 had the highest cochlear hair cell infection efficiencies when injected through the posterior semicircular canal. AAV2-7m8 also infected the supporting cells and spiral ganglion cells, albeit at lower levels. Application of AAV-GFPs through posterior semicircular canal injections resulted in minimal change in hearing. When applied on the round window membrane with a gelatin sponge, AAV2-7m8 was also able to infect cochlear hair cells, but the infection efficiency is lower than posterior canal delivery. **Conclusions:** Several novel AAV strains were tested in this study. We found that AAV2-7m8 was able to infect cochlear inner and outer hair cells at high efficiencies when injected through the posterior semicircular canals. In addition, AAV2-7m8 was capable of infecting cochlear hair cells when applied on the round window membrane with a gelatin sponge. These findings suggest AAV2-7m8 is an excellent viral vector for cochlear gene delivery.

703. Achieving High Yield of AAV Gene Therapy Vectors via Process Intensification

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Introduction: In recent years, AAV has emerged as one of the most promising gene delivery vectors in gene therapy. With time, there has been a recognizable advancement in AAV production technology which mainly includes mammalian cell-based, and insect cell baculovirus expression vector (IC-BEV) based platforms. However, a simple, scalable production process with a high yield of AAV vectors still represents a major challenge. In this research work, we are proposing IC-BEV based high cell density AAV5 production process using One Bac system. One Bac incorporates transformed Sf9 cell line harboring AAV rep and cap gene sequence which upon infection with a single baculovirus carrying transgene produces AAV. The scalability of IC-BEV platform and requirement of only single baculovirus with this system makes AAV production process simpler. **Method:** Transformed insect cell line was evaluated for stability of incorporated rep and cap expression over extended passage numbers and plaque purified working BV stock with the infective titer of 10^8 pfu/mL magnitude was generated for further work. Based on BV multiplicity of infection (MOI) data, an MOI of 3 was chosen for infection. In this fed-batch process, cells grown in serum-free medium were supplemented with a nutrient cocktail at specific time points of growth phase and post-infection phase. Cells were infected with a BV at 5 million cells/mL cell density, and the suspension culture was harvested 72-96 hours post infection. After process optimization experiments at shake flask level, the process was further validated at 1L bioreactor scale. **Results:** The cell line demonstrated stable expression of rep and cap gene up to 38 passage number. AAV production in batch mode resulted in $5.4x10^{10}$ vg/mL volumetric yield which was further increased to $9.5x10^{10}$ vg/mL in fed-batch mode at shaker flask level. AAV production by this fed-batch process in a bioreactor under controlled conditions provided $2x10^{11}$ vg/mL volumetric yield with a 4-fold and 2-fold increase in titer compared to the batch mode and fed-batch mode at shake flask respectively. The cell-specific yield at both scales was maintained during the batch and fed-batch mode of production. **Conclusion:** A fed-batch process employing high MOI BV infection at high cell density resulted in a high volumetric yield of AAV. Demonstration of process scalability at bioreactor scale displays considerable potential meet the demand of high quantity of AAV vectors during clinical studies.

704. Campaign-Ready Packaging System for Adeno-Associated Virus 1, 3b, 5, 8 and 9 Utilizing Two Plasmid

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A number of packaging systems are available for adeno-associated vectors (AAV). The most common is a triple transfection system in which one plasmid contains the inverted terminal repeats (ITR) s flanking the proviral construct, the second plasmid contains Rep and Cap genes and the third plasmid contains the Ad helper genes. While versatile, this approach has limitations, including the potential inefficiencies of multi-component transfection. Here we describe a system using a double plasmid transfection, in which Rep and Cap genes and Ad helper genes are on the same plasmid, pQT. We have created 5 different AAV cap packaging plasmids allowing us to package AAV1 (pQT-1), AAV3b (pQT-3b), AAV5 (pQT-5), AAV8 (pQT-8) and AAV9 (pQT-9). Using these plasmids, we have packaged GFP as a reporter gene from all 5 capsid variants, side by side with the traditional triple transfection system. Vector yields, as determined by digital PCR for genome copies and silver stains for vector particles, were comparable between double and triple transfection methods for all capsid variants tested, as were the empty-to-full capsid ratios, as judged by transmission electron microscopy. In addition, *in vivo* side by side comparison of triple and double transfection produced vectors were performed by intravenous injection via the tail vein for AAV3b, AAV5, AAV8 and AAV9, and intramuscular injection for AAV1 into C57/Bl6 mice. To determine efficiency of expression and transduction, immunohistochemistry of GFP staining and vector genomes will be evaluated in muscle and liver four weeks after injection. Lastly, optimization of double transfection packaging system was evaluated

by titrating different molar ratios of the two packaging plasmids, with a different transgene insert human Alpha-1-antitrypsin (AAT) gene. Further in vivo studies will be performed in Rag-/- mice for bioactivity of vectors by assaying AAT expression in the serum of mice injected IV AAV8 vectors packaged with different molar ratios. In conclusion, this data allows for mid-range (1E12-1E16) campaign ready packaging of AAV vectors AAV1, AAV3b, AAV5, AAV8 and AAV9.

705. Evaluation of the Safety and Efficacy of a Novel AAV Variant-Based *Ex Vivo* Cell Gene Therapy for Treating Hypoxic Ischemic Brain Injuries

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Recently, adeno-associated viral (AAV) vectors have been attracting attention in gene therapy research for use in treating various intractable diseases due to the characteristics that make them safe for treatment, such as their non-pathogenicity and replication deficiency. However, their limited tropism and innate immunogenicity have been barriers to expanding their applications. AAV capsid engineering could overcome these limitations. In this study, for preparing genetically enhanced hNSCs which could secrete interleukin-10 (IL-10) to improve the therapeutic effects in treating neonatal hypoxic-ischemia, the safety and efficacy of AAV variant "AAV r3.45" which was developed to enhance human neural stem cell (hNSC) transduction were evaluated. First, replication competent-AAV assays and contamination tests were performed on AAV r3.45 and other traditional AAV serotypes and the *in vivo* distribution of the delivered gene expression was analyzed to confirm its tropism. Second, the characteristics of AAV r3.45-infected hNSCs, such as apoptosis, transduction level, transduction duration, and gene insertion, were analyzed. Finally, the *ex vivo* therapeutic effects of the IL-10-secreting AAV-hNSCs were evaluated by *in vivo* animal model studies. This research could serve as the standard for the preparation of clinical trials for novel AAVs and AAV-mediated cell gene therapies.

706. AAV-Serotype-Specific Transduction Patterns in Mice and Non-Human Primates (NHPs) Liver Tissue: Implications for Therapeutic Efficacy

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AAV-based liver gene therapy has proven efficacious in mouse models of inherited disorders, but little is known about the transduction pattern of various AAV serotypes in the primate or human liver. To address this question, we assessed the AAV distribution pattern in the liver tissue of mice and non-human primates (NHPs) injected with either AAV serotype 1 or 5^{ch} (chimeric AAV5 in which VP1-unique portion is of AAV2 origin). The overall percentage of cells positive for the presence of AAV vector DNA/hFIX transgene RNA as well as the intensity and area of the positive signal were assessed. Additionally, AAV vector spatial distribution throughout the liver tissue was determined.
Methods:
C57BL/6 mice were injected intravenously (IV) with either AAV1-hFIX (human factor IX), $AAV5^{ch}$-hFIX at dose 1.46 e13 gc/kg or PBS, while NHPs were injected with either AAV1-hFIX, $AAV5^{ch}$-hFIX at dose 3e13 gc/kg or PBS. Liver tissues were collected post mortem and analyzed by fluorescent in situ hybridization (FISH) using fluorescent probes specific for AAV vector DNA and hFIX transgene mRNA. Hepatocytes were characterized based on Albumin RNA expression in mice and Serpina1 RNA expression in NHPs (FISH) while central veins were localized based on Glutamine Synthetase (GS) protein as determined by immunohistochemistry (IHC). FISH to detect Albumin RNA in mice or Serpina1 in NHPs, FISH for hFIX AAV vector DNA/transgene RNA and IHC for GS were performed on the same sections. Images were acquired with Aperio Versa 8 slide scanner (Leica Biosystems) and analyzed with the use of an image analysis software (HALO, indica labs). For FISH image analysis, cells were scored from weak positive **(1+)** to strong positive **(4+)** based on combination of average positive signal area [μm^2] and average intensity of positive signal within cell [RFU].
Results:
In mice the total percentage of liver cells positive for the presence of AAV vector DNA/hFIX mRNA was similar for AAV1 (Mean= 93.79 %; n=4) and $AAV5^{ch}$ (Mean= 96.36 %; n=5). However, liver cells positive for $AAV5^{ch}$ vector DNA/hFIX RNA displayed a higher signal score (% of 4+ cells: Mean=55.9 %; n=5) than liver cells positive for AAV1 vector DNA/hFIX RNA (% of 4+ cells: Mean=42.6 %; n=5) suggesting more efficient transduction per cell by the $AAV5^{ch}$ vector. The majority of cells transduced by AAV1 or $AAV5^{ch}$ (>99%) expressed Albumin and therefore were characterized as hepatocytes. Interestingly, the spatial distribution of AAV vector DNA/hFIX RNA positive signal within the liver tissue was different for the two serotypes: $AAV5^{ch}$ vector DNA/transgene RNA was more localized around the central vein whereas AAV1 was more homogenously distributed throughout the liver tissue. In NHPs, the percentage of liver cells positive for AAV vector DNA/hFIX RNA was higher for $AAV5^{ch}$ (Mean= 45.53%; n=2) than for AAV1 (Mean= 26.3 %; n=3), and similar to mice, $AAV5^{ch}$ resulted in a higher AAV vector DNA/hFIX RNA probe signal score (intensity and area of positive signal) indicating a more efficient transduction per cell with $AAV5^{ch}$ than with AAV1 in injected NHPs. Preliminary analysis of AAV vector spatial distribution throughout the NHP liver tissue demonstrates differences between mice and NHPs in AAV distribution pattern for $AAV5^{ch}$ vector as in NHPs $AAV5^{ch}$ vector DNA/transgene RNA was homogenously distributed throughout the liver tissue. In summary, we observed remarkable differences in AAV1 and $AAV5^{ch}$ transduction profiles in liver tissue of mice and NHPs. These results indicate that mouse models may have a limited value to predict the efficacy of liver-targeted AAV-based gene therapy, in particular in the context of development of therapies for metabolic disorders.

707. Rapid AAV Neutralizing Antibody Determination with a Cell-Binding Assay

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Recombinant adeno-associated virus (rAAV) vectors have been developed as a successful vector for both basic research and human gene therapy. However, neutralizing antibodies (NAB) against AAV capsids can abolish the AAV infectivity on the target cells and severely reduce the efficacy of AAV transduction. Such effects greatly limit the applications of rAAV vectors and absence of AAV NAB has become a prerequisite qualification for patients to be enrolled in gene therapy. Nevertheless, accurate assess AAV NAB has always remained a challenging task and the most trustworthy NAB assay is an in vivo assay, which is both costly and time-consuming. Here we developed a rapid assay based on the fact that AAV NAB inhibits rAAV binding to the host cell surface and NAB titers are directly related to the amount of AAV genomes binding to the target cells. By quantifying the AAV genomes on the target cells in the presence of anti-sera at varying dilution, AAV NAB can be subsequently determined. The titer determined by this new method correlates well with the classical transduction-based assays. Comparing to the classical transduction-based assays, our new method can be carried out with a 30 min binding assay without waiting for lengthy transduction outcome, which has been a major source of inaccuracy when a particular serotype transduces poorly in the assay cells. As such, the main advantage of our new assay is independent of transduction performance of AAV serotype in the target cells. In summary, the AAV-cell binding assay for NAB determination offers an alternative method for in vivo NAB assay.

708. Design of a Sf9-Based Stable Cells Line for the Production of Recombinant Serotype 3 Adeno-Associated Viral Vectors (rAAV3)

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The major drawback of the Baculovirus/Sf9 system for recombinant adeno-associated viral (rAAV) manufacturing is that most of the Bac-derived rAAV vector serotypes, with few exceptions, demonstrate altered capsid compositions and lower biological potencies. Previously, we have described a modified OneBac platform for a scale up production of rAAV5 and rAAV9 serotypes characterized by a significantly higher biological potency, even in a comparison with HEK293-manufactured rAAVs. In this report, we describe a design of Sf9-based stable cell line expressing AAV2 Rep and AAV3 Cap with optimized VP1:VP2:VP3 stoichiometry. The optimal capsid protein ratios were achieved using an attenuated Kozak sequence preceding initiation AUG codon of VP1. The resultant system yielded rAAV3 with infectivity similar to HEK293-derived rAAV3 vectors and could be potentially utilized for a scalable good manufacturing practice (GMP)-grade vector production.

709. The Application of Spectrophotometry for the Estimation of Genome Copies and Full/Empty Ratios of Adeno-Associated Virus Particles

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Polymerase Chain Reaction (PCR) is the most common technique for determining the genome content of adeno-associated viruses (AAV), and Analytical Ultracentrifugation (AUC) is commonly used for estimating the level of empty capsids (containing only the protein capsids with no DNA) and full capsids (containing both protein and DNA). However, both techniques require multiple steps with complicated procedures that are time-consuming and relatively low-throughput, and a more efficient technique is desired for sample screening and in-process samples. Spectrophotometry is a common method for measuring concentration of products with known extinction coefficients at specific wavelengths, widely used to determine the protein content for therapeutic proteins. AAV content is more complicated to determine through spectrophotometry than many other therapeutic products because they contain two major species absorbing at different wavelength maxima (protein capsids at 280 nm, and DNA at 260 nm), and because capsid particles contain a heterogeneous mixture of empty and full capsids. However, determining AAV content directly by spectrophotometry is possible without prior sample treatment based on equations derived to determine both the level of genome copies and total capsids per sample, and these equations can be further utilized to calculate the ratio of empty and full capsids. Due to the low concentration of AAV products, matrix interference from buffer components and silicone leaching from vial containers can generate a large impact on the spectra profile and AAV content determination. For such samples, the spectrophotometric method was further optimized with filtration steps to capture the matrix components to subtract out the absorbance interference. The method was demonstrated to be precise and linear. Genome values calculated by spectrophotometry have good correlation to values determined by the PCR method. The estimated distribution of empty and full capsids calculated by spectrophotometry also correlate well with AUC values. In summary, spectrophotometry combined with baseline matrix interference subtraction is a faster and high-throughput alternative to the labor-intensive methods commonly used for AAV analysis for the determination of genome and capsid content.

710. Tracking ABCD1 Expression Using HA-Tagged ABCD1 Following Intrathecal AAV9 Delivery

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Mutations in the gene encoding the peroxisomal ATP binding cassette transporter (ABCD1) cause elevations in very long chain fatty acids (VLCFA) and the neurodegenerative disease adrenoleukodystrophy. Previously we showed that gene correction in the Abcd1-/- mouse after recombinant AAV9-CBA-hABCD1 intrathecal delivery led to a 30% decrease of VLCFA levels in spinal cord. However, this gene and lipid correction was not achieved in dorsal root ganglia (DRG). To optimize detection of ABCD1 and better understand its biodistribution, we developed a method using hemagglutinin (HA)-tagged ABCD1 to track ABCD1 following AAV9 delivery. A C-terminal HA-tagged human ABCD1 cDNA was cloned into pAAV-CBA vector and packaged into rAAV9 in 293T cells. When we treated 293T cells with AAV9-ABCD1-HA ($1X10^4$gc/cell and $1X10^5$gc/cell), there was a dose-dependent increase of ABCD1 protein expression; meanwhile, a strong signal was also detected using the HA-tag antibody, suggesting successful tagging of the ABCD1 protein. To confirm the localization of ABCD1 with HA-tag *in vitro*, we assessed mixed brain glial cells from postnatal mice treated with AAV9-ABCD1-HA at $5X10^5$gc/cell for 5 days. Immunostaining demonstrated exact co-localization of ABCD1 with the HA-tag. To corroborate the feasibility of using AAV9-ABCD1-HA to track ABCD1 *in vivo*, we carried out intrathecal delivery of AAV9-ABCD1-HA via an osmotic pump (IT pump). Tissues were harvested after 15 days of injection and then fixed for immunostaining using an HA-tag antibody. The results showed that delivery via IT pump led to extensive expression of ABCD1-HA across different cell types in the spinal cord but was largely limited to neurons in DRGs. The normal endogenous pattern of ABCD1 seen in wild type DRG satellite cells was not achieved. To assess for functionality after AAV9-hABCD1 delivery, we measured VLCFA across different cell types *in vitro*. To our surprise, we could correct VLCFA levels in mixed brain glial cells but not in isolated DRG satellite cells. Cells treated with HA-tagged-ABCD1 were also collected for VLCFA analysis. In conclusion, AAV9-ABCD1-HA provides a more reliable way for tracking ABCD1 distribution after *in vivo* delivery. Compared with the less reliable ABCD1 antibody staining, HA-tag staining provides higher specificity and demonstrates that AAV9-ABCD1-HA localizes mainly to neurons in DRG. Studies are pending to assess whether the HA tag could interfere with normal ABCD1 function.

711. Large Scale Production and Analysis of Ancestral AAV Generated Using Baculovirus Based System

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The use of viral vectors for gene therapy continues to receive significant attention as more viral gene therapy products advance into the clinic. With the approval of the first AAV based product in the US and over 120 active/recently completed clinical studies involving recombinant AAV (rAAV) gene therapy products worldwide, there is an increasing demand for scalable GMP production strategies that can produce sufficient material to support late phase clinical trials and commercialization. While transient transfection of mammalian cells remains the most commonly used process (although not scalable), we, at Lonza, have been able to scale up the baculovirus-based platform and Producer cell line based processes to 100L and 2000 L scales respectively. Recent advances in the field have led to development of Ancestral AAV vector (Anc80), a predicted ancestor of AAV 1, 2, 8 and 9. Anc80 shows efficient transgene delivery in murine models and some non-human primates with no notable signs of toxicity, low levels of cross-reactivity and reduced neutralization *in vivo.* These attributes suggest that Anc80 may address the challenges associated with pre-existing immunities associated with other AAV serotypes. Given the clinical advantages of Anc80 and our expertise with generating rAAV using the rBV platform in insect cells, we have developed a system for producing Anc80AAV. Using previously established technology, we generated bacmid DNA and rBVs containing Anc80. Western blot confirmed expression of capsid proteins (VP1, VP2 and VP3) as well as the viral regulatory proteins (Rep78, -68, -52 and -40). Following amplification and titration of the baculovirus, Anc80 AAV-GFP was produced by co-infecting cells with Anc80 and GFP rBVs in small scale STRs. Western blot analyses of clarified cell lysates showed expression of rep and cap proteins and AAV titers up to 7E10 vg/ml. Following a 2-column purification scheme, characterization of the titer, identity, purity, infectivity and full/empty ratios were determined. Using the baculovirus expression system, we are able to generate Anc80 with negligible loss of productivity during the scale up from 0.02 to 3L and further scale up to 50L bioreactor scale. Ongoing work includes in vitro analysis of infectivity and gene transfer of Anc80AAV generated using recombinant baculovirus technology. Through the development of this process, our goal is to improve productivity and scalability while reducing development costs and shortening timelines. Data from optimization, scale up and comparison of rAAV generated using rBV based platform with other production systems will be presented.

712. Formulation Optimization for a Gene Therapy Parenteral Product

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Gene therapy product stability is dependent on the selection of the right excipients. An optimal formulation may enhance drug product stability by preserving the native conformation of the capsid proteins and subsequently preventing nanoparticle aggregation. AT132 is an AAV8 nanoparticle containing a myotubularin expression cassette for the treatment of X-Linked myotubular myopathy. The current study details experiments undertaken to optimize the formulation for AT132 in order to support all

functions starting from manufacturing to delivery to the patients. Biophysical techniques were used to evaluate the colloidal stability of AT132 when exposed to heat stress, which can lead to protein denaturation and subsequent formation of aggregates. Particle aggregation can be monitored by changes in size, intensity, and polydispersity by dynamic light scattering (DLS). The initial buffer screen covered the range from pH 2.5 to pH 9.5. There were two distinct phases observed in the temperature profiles: an early phase (T onset) where a consistent small change in the average size was observed, followed by a more significant size increase (more than 10X) in the particle sizes which was characterized by the point at which ~50% of the size transition had occurred (Tm). The Lactated Ringer's Solution (LRS) formulation proved to be one of the best formulations in this first screen. The next set of experiments explored whether additional excipients (e.g. histidine or $MgCl_2$) could increase the thermal stability of AT132. While the data indicated that $MgCl_2$ may have increased the thermal stability of AT132, processing concerns such as potential for protease activation or buffer salt precipitation precluded the addition of $MgCl_2$ to the LRS formulation of AT132. Additional real-time studies at multiple temperatures demonstrated that LRS containing 0.01% Pluronic F-68 was a good formulation for AT132.

713. Key Rep-Proteins Necessary for the Adeno-Associated Virus Production by Transient Transfection in HEK293 Cells in Suspension and Serum-Free Medium

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Introduction: Adeno-associated virus (AAV) is proving to be a powerful tool for gene transfer in gene therapy applications. As of January 2018, 204 clinical trials used AAV as a tool for gene transfer which ranks it as number 4 amongst all the vectors used for gene therapy applications. **Methodology:** One of the most common methods of AAV production is by adenovirus-free transient transfection of HEK293 cells with plasmids carrying necessary components for vector assembly and gene of interest (GOI). Adenovirus components are supplied by the stably integrated E1A and E1B genes present in the 293 cell line, and other components, such as, E4, E2A and VAI, are provided by transient transfection along with Rep/Cap and GOI. The Rep gene encodes four Rep proteins (Rep 78, 68, 52, 40) produced by activation of p5 and p19 promoters by the proteins expressed by E1A gene present in 293 cells. The relative importance of each Rep for AAV production is not fully understood. We report here the key Rep proteins that are necessary for the AAV production by first generating the four plasmids expressing individual Rep proteins and later producing AAV with various combinations of these plasmids along with Capsid and Adeno-helper plasmids. Because high level of expression of Rep could be toxic to the cells, we control their expression using the Cumate inducible promoter (CMV5CuO), which is repressed by the presence of the cumate repressor (CymR). The p19 is located within the coding region of Rep78 and Rep68, therefore this sequence was modified without changing the amino acid sequence to prevent expression of Rep52 and Rep40. To determine which Rep proteins are essential, a transient transfection was done using a combination of plasmids encoding Rep78, 68, 52, and 40. HEK293 cells and HEK293 cells expressing the CymR repressor (HEK293-CymR) were used for these experiments. **Results and Conclusions**: We showed that none of the Rep expressed individually was capable to produce AAV. A combination of one large Rep (68 or 78) with one small Rep (52 or 40) was sufficient to produce rAAV. The minimum of two Reps (68 and 40) were able to generate high titers of AAV. AAV titers were higher when HEK293CymR were used for transfection in the absence of cumate (Off situation). Taken together these data indicate that it might be possible to increase the production of rAAV by transient transfection by optimizing the quantity of Rep produced by the cells.

714. Refinements of rAAV8 Purification Protocol with Chromatography Technology

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Background: Recombinant adeno-associated viruses (rAAVs) generated by each natural AAV serotypes display different tissue tropisms *in vivo*. Therefore, rAAVs have emerged as a versatile delivery vehicle for gene therapy. Actually, rAAV-based drugs have already been approval of gene therapy. In order to extend the possibility of rAAV-based gene therapy in human, it is essential to improve the methodologies for purification of rAAV. The purification process is preferred to be cost-effective and reproducible for mass production, especially for human gene therapy. Conventional purification methods using density gradient ultracentrifugation for rAAV are not suitable for large-scale purification. Therefore, the development of alternative rAAV preparation method is necessary to satisfy the above requirements. Chromatography-based purification strategy is more suitable for large-scale vector purification than commonly used ultracentrifugation-based methods. We have recently developed efficient protocols for rAAV1 and rAAV9 preparation by chromatographic purification without ultracentrifugation-based approach. Contrary to rAAV1, rAAV9 particles are unlikely to bind efficiently to ion-exchanger at certain condition. Therefore, the purification method is different between serotypes. In this study, we tried to elucidate the purification condition of rAAV8, which is used for gene therapy of hemophilia and muscular dystrophy. **Methods:** rAAV8 (scAAV8-CBA-AcGFP1) was produced by the triple-transfection to HEK293EB cells in no serum medium. The cultured medium was harvested five days after transfection and concentrated by the Tangential Flow-Filtration (TFF, exclusion limit is 750 kDa). Subsequently, heat treatment, saturated ammonium-sulfate precipitation and chromatographic purification were performed. **Results and conclusion:** Contaminated lower-molecular-weight proteins were removed by heat treatment. We succeeded to reduce further contaminants by ammonium-sulfate precipitation. We found the condition that almost rAAV8 was bound to cation-exchange columns efficiently. The protocol may be applicable to other serotypes except rAAV9.

715. AAV Vectors are Going Viral for Application in Gene and Cell Therapy

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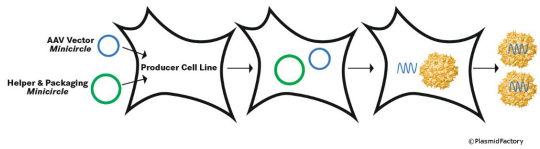
Although gene and cell therapy are promising tools for the treatment of genetic diseases, progress has been slow in developing effective clinical approaches. The issue lies in the difficulty to develop safe and efficient gene-delivery systems. Amongst other applications, plasmid DNA is often used as starting material in the GMP-compliant production of recombinant viruses, antibodies and RNA. Therefore, it also represents a solid start for adeno-associated viruses (AAV) vector manufacturing, where these vectors are the active pharmaceutical ingredients (API) used in clinical trials. There are several opportunities to produce efficient and pure AAV vectors. For example, the choice of the serotype and of the applied plasmid system is important. Replacing the plasmid system by minicircle DNA allows to reduce contaminations and to increase transduction efficiencies of scAAV preparations significantly. We will give an overview regarding the first important steps of AAV vector manufacturing.



716. Structural Protein of Various AAV Serotypes is Involved in the Transcription of Viral Genome

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Introduction: The amino acid Y704 in adeno-associated virus serotype 2 (AAV2) capsid is near the 2-fold interface. We previously reported that mutation of this surface-exposed tyrosine residue to phenylalanine (AAV2-Y704F) could modestly increase the recombinant AAV2 (rAAV2) vector-mediated transgene expression (***PNAS.*** 2008; 105(22):7827-32.). Later, similar results were obtained using other AAV serotypes (***Gene Ther.*** 2012; 19(4):375-84.). Interestingly, Dr. Muzyczka's group observed that mutation of the same position to an alanine residue (AAV2-Y704A) was defective for transcription of the packaged viral genome (***J Virol.*** 2014; 88(2):1071-9.). Briefly, the mutant virus particles were able to enter the target cell, travel to the nucleus, uncoat, and synthesize a second strand, whilst they were unable to transcribe messenger RNAs (mRNAs). It is worth noticing that all infections using AAV2-Y704A vectors were conducted in the presence of adenovirus to simulate a normal productive infection coinfection. In this report, we would like to characterize whether the mechanistic roles of the Y704 in vector-mediated transgene expression might differ among AAV serotypes in the absence of helper adenovirus. **Methods and Results:** By site-directed mutagenesis, we generated pairs of point mutants on various AAV serotypes: AAV2-Y704A, AAV2-Y704F; AAV3-Y705A, AAV3-Y705F; and AAV6-Y705A, AAV6-Y705F. It was concluded that all the mutant capsids encapsidate viral genomes at a similar level. Then, *in vitro* cell binding and internalization assays were performed by determining the number of total viral genomes present on the cell membrane or inside the cell, respectively. Control experiments confirmed that Trypsin digested more than 90% of AAV particles when bond to cell membrane and that Trypsin treatment had no effect on the internalized viral particles. Our results demonstrated that similar to AAV2, all other mutant capsids have little effect on viral binding and internalization ability. In terms of intracellular trafficking, Y to A mutants had a similar ability compared to the wild-type capsid, whilst Y to F mutants appeared to increase it. On the other hand, all the Y to A mutants dramatically shutdown the mRNA transcription and hence the transgene expression *in vitro*, whilst most Y to F mutants significantly increased both the mRNAs and proteins. To our surprise, we found that the defective effect of Y to A mutants on transgene expression is not only *in cis* manner, but also *in trans*. Further mechanistic studies indicated that the inverted terminal repeat (ITR) of viral genome might play a significant role. Next, an additional pair of viral vectors, AAV8-Y707A and AAV8-Y707F will be generated to test our hypothesis in mice liver *in vivo*. **Conclusion:** Here, we have characterized the biological properties of Y704 in AAV capsids and concluded that they are conservative among AAV serotypes. Interestingly, the Y to A mutants failed to accumulate mRNA transcripts in the presence of significant levels of viral ITR-containing DNAs in the nucleus.

Adenovirus Vectors and Other DNA Virus Vectors

717. Delivery of a Multiplex CRISPR/Cas9 Machinery for the Treatment of Duchenne Muscular Dystrophy with One Single Gene Deleted Adenoviral Vector

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Recent advances in CRISPR/Cas9 based genome editing enable the correction of mutations causing Duchenne muscular dystrophy (DMD) by developing personalized treatments for the different mutations underlying DMD. Recent studies showed efficient in vivo genome editing following AAV delivery of a DMD specific CRISPR/Cas9 machinery. Nevertheless viral delivery of all required CRISPR/Cas9 components including Cas9 together with multiple guide RNA (gRNA) expression units using one single vector has not been fully exploited yet. Gene deleted high-capacity adenoviral vectors (HCAdVs) offer the packaging capacity to deliver the complete CRISPR/Cas9 machinery including several gRNA expression units. Here we present a new

toolbox that facilitates customization of the CRISPR/Cas9 system and subsequent cloning and production of CRISPR-HCAdVs containing a Cas9 gene and multiple gRNA expression units. We produced a CRISPR-HCAdV containing two gRNAs specific for intronic sequences flanking DMD exon 51. Purified DMD specific CRISPR-HCAdV where used to transduce immortalized dystrophic skeletal muscle cells derived from a DMD patient with a Δexon 48-50 mutation leading to frameshift and premature stop codon in DMD exon 51 and absence of full length dystrophin protein. Upon adenoviral gene transfer followed by DMD specific CRISPR/Cas9 expression locus specific PCR and sequencing of deletion specific CPR products confirmed efficient locus specific deletion of DMD exon 51 on genomic level. Sequencing of deletion specific RT-PCR products revealed seamless splicing of DMD Exon 47 to exon 52 on mRNA level. Fluorescent imaging DMD expression showed successful reconstituted DMD expression after treatment of dystrophic muscle cells with a Δexon 48-50 mutation. Our results show that CRISPR-HCAdVs are efficient delivery vehicles for CRISPR/Cas9 to support gene editing in the context of DMD. Our delivery approach broadens the opportunities for potential in vivo delivery of CRISPR/Cas9 for DMD gene editing and will be further explored in preclinical studies. As our CRISPR/Cas9-HCAdV production pipeline can be easily adapted to incorporate other or even more gRNA expression units, it provides a valuable platform to develop personalized CRISPR-HCAdV for the treatment of individual DMD mutation for every single patient. Moreover we tested 20 different Adenovirus serotypes for their efficiency to transduce muscle cells using our reporter tagged Adenovirus library. AdV serotypes 35, 37 and 21 showed muscles cell transduction efficiencies superior to AdV5 that is commonly used as gene therapy vector. These serotypes are promising candidates for the conversion into highly efficient vectors for gen therapeutic treatments of various muscular diseases including DMD.

718. Efficient Systemic Treatment with Mesothelin-Targeted Oncolytic Adenovirus in Patient-Derived Xenograft (PDX) Model of Pancreatic Cancer

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Pancreatic cancer is an aggressive malignant disease. Despite extensive efforts, systemic therapies have provided only limited efficacy for patients with this disease. Oncolytic Adenovirus (OAd) is a promising therapeutics, and it is also known for its efficient *in vivo* gene delivery. However, when adenovirus vectors are injected intravenously into mice, most of the virus goes to the liver and can lead to liver toxicity at high dosage. One of the reason for liver tropism is that hepatocytes express high levels of the primary adenovirus receptor, and non-parenchymal liver cells, such as Kupffer cell and epithelial cell, also capture the viral particle. As a consequence of large sequestration of adenovirus by liver, the tumor transduction rate is low and the in vivo efficacy is limited. Therefore, the improvement of cancer selective transduction and vector distribution to avoid liver sequestration would overcome the obstacles for systemic delivery required for efficient systemic treatment of spread and/or metastatic lesions of pancreatic cancer with OAd. To improve the tumor transduction, we have generated the pancreatic cancer-targeted OAd by high-throughput screening of Ad-fiber library in mesothelin (MSLN) expressing cells. The pancreatic cancer-targeted OAd binds to MSLN protein, which is overexpressed on the surface of pancreatic cancer. MSLN-targeted OAd showed selective and powerful anti-tumor effect against Panc-1 xenograft tumor model in both intratumoral (i.t.) and intravenous (i.v.) injection. Importantly, when we assessed viral distribution after i.v. injection, the liver sequestration of MSLN-targeted OAd was lower than untargeted OAd (Ad5 WT virus) at 48 hrs after injection. By day 7, the viral copy number of MSLN-targeted OAd in the tumor was significantly higher than Ad5 WT virus. These results suggest that systemic injection of the tumor targeted-OAd showed significantly lower liver sequestration and better tumor accumulation. Additionally, we performed multiple time point injection of MSLN-targeted OAd against regrown tumors. Four out of six tumors were controlled with repeated injection. Next, antitumor effect of MSLN-targeted OAd was assessed in patient-derived xenograft (PDX) model. After intravenous administration, only the MSLN-targeted OAd showed significant antitumor effect compared to the untreated group ($p<0.05$), while the growth of Ad5 WT virus injected group was same as untreated group. In this study, systemic injection of MSLN-targeted OAd showed remarkable anti-tumor effect in both systemic and intratumoral injections at low dose. Our results indicated that tumor targeted-OAd can embody efficient systemic treatment for pancreatic cancer which are mostly found with spread or metastatic lesions.

719. Adenoviral Vectors Armed with HPV Oncogene Specific CRISPR/Cas9 for Specific Tumor Gene Therapy of HPV Related Cancers

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Different types of human Papillomaviruses (HPV) cause malignant epithelial cancers including cervical carcinoma, non-melanoma skin cancer as well as head and neck cancer. The HPV oncoproteins E6 and E7 inhibiting apoptosis and driving cell cycle progression are responsible for tumor development. It was shown that HPV oncogene disruption using CRISPR/Cas9 induces apoptosis and cell cycle arrest. However no attempts were made to translate these promising findings towards in vivo applications using viral delivery of the HPV specific CRISPR/Cas9. By using our improved toolbox that facilitates customization of the CRISPR/Cas9 system and subsequent cloning and production of CRISPR/Cas9 expressing adenoviral vectors we constructed gene deleted adenoviral vectors (HCAdVs) and E1, E3 deleted AdV armed with CRISPR/cas9 machinery that is specific for the major high risk HPV types HPV16 and HPV18. Cervical cancer cell lines Siha and Hela containing HPV16 or HPV18 genomes integrated into the cellular genome as well as HPV negative A549 lung cancer cells were transduced with HPV specific CRISPR-HCAdVs or CRISPR-AdV respectively. Expression of HPV type specific CRISPR/Cas9 upon adenoviral delivery resulted in cell death in HPV positive cervical cancer cell lines whereas HPV negative A549 cells were unaffected. Transduced cervical cancer cells showed increased apoptosis induction and decreased proliferation and viability compared to untreated or

HPV negative control cells. This suggests that AdV and HCAdVs can serve as HPV specific cancer gene therapeutic agents when armed with HPV specific CRISPR/Cas9. Because of the versatility of the CRISPR/Cas9 system and new flexibility in constructing CRISPR-AdV we hope that our approach can contribute to establish complementary personalized treatments specific for the respective HPV type present in each individual tumor.

720. S/MAR Based Nano-Vectors: a Novel Non-Viral, Non-Integrative System for the Efficient Genetic Engineering of Cells

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We have previously demonstrated that we can generate genetically modified cell-lines using autosomally replicating S/MAR DNA vectors. Some cell types such as primary human and murine cells are typically refractory to transfection with traditional plasmids that contain a bacterial backbone which comprises an antibiotic resistance marker and the pUC replication origin. To overcome one aspect of the toxicity which can be derived from the bacterial sequences contained in these vector systems, we have generated next generation S/MAR DNA vectors based on the Nanoplasmid ™ vector system developed by Nature Technology Corporation to generate a novel DNA vector platform suitable for the genetic engineering of cells. This novel DNA Nano-Vector system is characterized by a minimally sized bacterial backbone and an antibiotic free RNA-Out selection system that allows its manipulation and expansion in a specially engineered strain of *E.coli*. The manufacturing of these plasmids generates a high yield of supercoiled DNA by normal DNA preparation without the need of genetic recombination events and/or additional steps of purification as with Minicircle vectors.The presence of the S/MAR region prevents epigenetic silencing and provides persistent mitotic stability. We show that S/MAR Nano-Vectors are more efficient with improved efficiency in establishing stable cells when compared to the respective vector with a bacterial backbone.Nano-S/MARt vectors routinely provide more robust transgene expression than standard plasmid DNA, and present an increased mitotic stability over hundreds of cell divisions. We demonstrate their application in generating persistently modified Patient Derived Pancreatic Cancer Cells (PDX), primary mouse embryonic stem cells (mESC), mouse embryonic fibroblasts (MEF) and human primary cells.To demonstrate that the introduction of a Nano-SMARt vector does not affect the molecular and biochemical behavior of the cells we have performed microarray studies on cells labelled with the reporter gene GFP and we compared their transcriptome profiles to parental unmodified cells. We show that the number of transcripts perturbed by the presence of this novel class of DNA Vectors is minimal which demonstrated that cells modified with this DNA vector system are essentially isogenic.We have used this novel DNA vector system to generate isogenic tumor models which provide insight into the mechanism of pancreatic cancer development by restoring the crucial tumor suppressor gene SMAD4 to Patient Derived Pancreatic Cancer cells (PDX) without altering the molecular or biochemical integrity of the cells with vector mediated toxicity. Patient Derived Pancreatic Cancer Cells were modified using the Nano-SMARt vector system and by comparing the growth characteristics and histology of the cells we demonstrate that the introduction of the vector does not affect their behaviour. In subsequent experiments we restored the key tumour-suppressor gene SMAD4 which had been lost during the malignant transformation of the pancreatic cancer cells. The success of the re-introduction of the gene was determined in vivo in xenograft models. The tumor phenotype was rescued in cells in which SMAD4 functionality was restored as they failed to form pancreatic tumors upon engraftment whereas unmodified cells and cells labelled with the reporter gene GFP generated identical well differentiated ductal pancreatic adenocarcinomas. We show that this novel class of DNA Vectors can be used to persistently modify a wide range of cells providing sustained and high levels of transgene expression, whilst avoiding the risk of vector mediated toxicity and insertional mutagenesis.

721. Abstract Withdrawn

722. Oncolytic Adenovirus Regulated by Tumor Microevnrionment-Targeted Hybrid Promoter Elicits Potent Antitumor Effect against Desmoplastic Pancreatic Cancer

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Pancreatic cancer is a leading cause of cancer-related death. Desmoplastic pancreatic tumors exhibit excessive extracellular matrix (ECM) and are thus highly resistant to anticancer therapeutics, since the ECM restricts drug penetration and dispersion. Here, we designed and generated two hypoxia-responsive and cancer-specific hybrid promoters, H(mT)E and H(E)mT. Transgene expression driven by each hybrid promoter was markedly higher under hypoxic conditions than normoxic conditions. Moreover, H(E)mT-driven transgene expression was highly cancer-specific and was superior to that of H(mT)E-driven expression. A decorin-expressing oncolytic adenovirus (Ad; oH(E)mT-DCN) replicating under the control of the H(E)mT promoter induced more potent and highly cancer-specific cell death compared with its cognate control oncolytic Ad, which harbored the endogenous Ad E1A promoter. Moreover, oH(E)mT-DCN exhibited enhanced antitumor efficacy compared with both the clinically approved oncolytic Ad ONYX-015 and its cognate control oncolytic Ad lacking DCN. oH(E)mT-DCN treatment also attenuated the expression of major ECM components, such as collagen I/III, elastin and fibronectin and induced tumor cell apoptosis, leading to extensive viral dispersion within orthotopic pancreatic tumors and pancreatic cancer patient-derived tumor spheroids. Collectively, these findings demonstrate that oH(E)mT-DCN exhibits potent antitumor efficacy by degrading the ECM and inducing apoptosis in a multifunctional process. This process facilitates the dispersion and replication of oncolytic Ad, making it an attractive candidate for the treatment of aggressive and desmoplastic pancreatic cancer.

723. Hepatocellular Carcinoma-Targeting and Promoter-Modified Oncolytic Adenovirus Overcomes Hypoxic Tumor Microenvironment and Effectively Disperses through Orthotopic Tumor via Systemic Injection

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Cancer-specific promoter driven replication of oncolytic adenovirus (Ad) is cancer-specific, but shows low transcriptional activity. Thus, we generated several chimeric α-fetoprotein (AFP) promoter variants, containing reconstituted enhancer and silencer regions, to preferentially drive Ad replication in hepatocellular carcinoma (HCC). Modified AFP promoter, containing 2 enhancer A regions and a single enhancer B region (a2bm), showed strong and HCC-specific transcription. In AFP-positive HCCs, gene expression was 43- to 456-fold higher than those of control AFP promoter lacking enhancers. a2bm promoter was further modified by inserting multiple hypoxia-responsive elements (HRE) to generate Ha2bm promoter, which showed stronger transcriptional activity than a2bm promoter under hypoxic conditions. Ha2bm promoter-regulated oncolytic Ad (Ha2bm-d19) showed a stronger antitumor and proapoptotic effect than did a2bm promoter-regulated oncolytic Ad (a2bm-d19) in HCC xenograft tumors. Systemically administered Ha2bm-d19 caused no observable hepatotoxicity, whereas control replication-competent Ad, lacking cancer specificity (d19), induced significant hepatic damage. Ha2bm-d19 caused significantly lower expression of interleukin-6 than d19, showing that HCC-targeted delivery of Ad attenuates induction of the innate immune response against Ad. This chimeric AFP promoter enabled Ad to overcome the hypoxic tumor microenvironment and target HCC with high specificity, rendering it a promising candidate for the treatment of aggressive HCCs.

724. Combinatorial Effect of Ing4 and Trail Gene Expressing Oncolytic Adenoviruses in Hepatocellular Carcinoma Combinatorial Effect of Ing4 and Trail Gene Expressing Oncolytic Adenoviruses in Hepatocellular Carcinoma

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Current treatments of hepatocellular carcinoma (HCC) are ineffective and unsatisfactory in many aspects. Cancer-targeting gene virotherapy using oncolytic adenoviruses (OAds) armed with anticancer genes has shown efficacy and safety in clinical trials. Nowadays, both inhibitor of growth 4 (ING4); as a multimodal tumor suppressor gene, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL); as potent-apoptosis inducing gene, are experiencing a renaissance in cancer gene therapy. Herein we investigated the antitumor activity and safety of mono-and combined therapy with OAds armed with ING4 (Ad-ΔB/ING4) and TRAIL (Ad-ΔB/TRAIL) gene, respectively, on preclinical models of human HCC. OAd-mediated expression of ING4 or TRAIL transgene was confirmed. Ad-ΔB/TRAIL and/or Ad-ΔB/ING4 exhibited potent killing effect on human HCC cells (HuH7 and Hep3B) but not on normal liver cells. Most importantly, systemic therapy with Ad-ΔB/ING4 plus Ad-ΔB/TRAIL elicited more eradicative effect on an orthotopic mouse model of human HCC than their monotherapy, without causing obvious overlapping toxicity. Mechanistically, Ad-ΔB/ING4 and Ad-ΔB/TRAIL were remarkably cooperated to induce anti-tumor apoptosis and immune response, and to repress tumor angiogenesis. This is the first study showing that concomitant therapy with Ad-ΔB/ING4 and Ad-ΔB/TRAIL may provide a potential strategy for HCC therapy and merits further investigations to realize its possible clinical translation.

725. Evasion of Antiviral Immune Response and Improvement in Tumor-Specific Localization of Oncolytic Adenovirus by Hydrogel System

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Currently, intratumoral injection of an oncolytic adenovirus (Ad) is the conventional administration route in clinical trials. Nonetheless, the locally administered Ad disseminates to the surrounding nontarget tissues and has short biological activity due to immunogenicity of Ad, thus necessitating multiple injections to achieve a sufficient therapeutic index. In the present study, a tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-expressing oncolytic Ad (oAd-TRAIL) was encapsulated in a gelatin hydrogel matrix (oAd-TRAIL/gel) to enhance and prolong antitumor efficacy of the virus after a single intratumoral injection. oAd-TRAIL/gel showed greater antitumor efficacy than naked oAd-TRAIL did due to enhanced and prolonged intratumoral accumulation of Ad up to a 20-day period, showing potent induction of apoptosis and inhibition of tumor cell proliferation. Furthermore, the gel system effectively prevented shedding of oncolytic Ad from the injection site to hepatic and other healthy tissues. oAd-TRAIL/gel treatment resulted in a markedly weaker antiviral immune response against Ad relative to naked oAd-TRAIL, further contributing to prolonged persistence of the oncolytic Ad in tumor tissue. Moreover, the hydrogel matrix preserved oAd-TRAIL's ability to induce an antitumor immune response, resulting in higher intratumoral infiltration by $CD4^+$/$CD8^+$ T cells. Taken together, these findings show that single intratumoral administration of the Ad/hydrogel system may prolong and potentiate the therapeutic efficacy of Ad, modulate the immune reaction in favor of the virotherapy, and enhance intratumoral localization of the virus, ultimately overcoming limitations of oncolytic virotherapy revealed in recent clinical trials.

726. Inhibition of Mortalin Leads to Anti-Fibrosis and Apoptosis of the Keloid Spheroid

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Mortalin is a mitochondrial chaperone of the heat shock protein 70 family and it's pro-proliferative and anti-apoptosis functions could be associated with keloid pathogenesis, and blocking of mortalin and its interaction with p53 might be a potential novel target for the treatment of keloid. Therefore, we generated mortalin-specific small hairpin (sh) RNAs (dE1-RGD/GFP/shMot) and introduced into keloid spheroids for examination of its apoptotic and anti-fibrotic effect. On keloid tissues, mortalin expression was higher than adjacent normal tissues and it's protein expressions were activated keloid fibroblasts (KFs). After primary keloid spheroid were transduced with dE1-RGD/GFP/shMot for knockdown of mortalin, expression of type I, III collagen, fibronectin, and elastin was significantly reduced and transforming growth factor-ß1, epidermal growth factor receptor (EGFR), Extracellular Signal-Regulated Kinases 1 and 2 (Erk 1/2), and Smad 2/3 complex protein expression were decreased. In addition, increased TUNEL activities and cytochrome C were observed. Further, for examine of mortalin and p53 interaction, we performed immunofluorescence analysis. Knockdown of mortalin relocated p53 to the cell nucleus in primary keloid spheroids by dE1-RGD/GFP/shMot transduction. These results support the utility of knockdown of mortalin to induce apoptosis and reduce ECMs expression in keloid spheroid, which may be highly beneficial in treating keloids.

727. Engineered Phage Matrices Facilitate Angiogenic Differentiation of Adipose Derived Stem Cells

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Although stem cell niche plays a vital role in stem cell differentiation towards different lineages, an artificial stem cell niche achieved so far is not successful to fulfill the complex microenvironment of the stem cell. Here, we demonstrated engineered hybrid phage matrices that possess cell adhesive and angiogenic peptides with a suitable scaffold by formulating polyacrylamide hydrogel incorporating phage in different stiffness to guide adipose derived stem cells (ASC) and could achieve higher stiffness favoring osteogenesis and lower stiffness favoring adipogenesis. In this study, we present a specific phage based angiogenic matrices by modulating physical and biochemical cues in differentiation of ASC, providing convenient artificial stem cell niche.

728. Engineered Phage Nanofibers Induce Angiogenesis

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Angiogenesis can be considered a hallmark for healing wounds and curing various ischemic diseases because therapeutic opportunities can be envisaged by achieving a vascular niche. Recently, nanofibrous structures of M13 phages have been considered as ECM-mimicking nanofibers, and have been used after chemical or genetic modification with cell signaling peptides as novel tissue engineering matrices to direct the desired cellular functions. Here, we employed bioinspired M13 nanofibers comprising extracellular and vascular niches and investigated their potential in inducing neovascularization as a treatment for ischemic diseases. An engineered phage nanofiber (expressing SDKP and RGD) can act as an angiogenic factor and an extracellular component at the same time, if we display angiogenic and/or integrin binding peptides together on its body, thereby providing phage angiogenic niches, and interacting with endothelial cells to induce angiogenesis. We found that cell viability, migration, elongation, and angiogenesis were predominantly affected by nanofibrous structures (topological cues) of the engineered phages, providing a therapeutic platform for providing therapeutic biochemical cues using specific peptide expression on phage coat proteins. The phage structure has many advantages for creating a therapeutic platform, that is, "a niche. Our engineered phage nanofiber provides an angiogenic niche with therapeutic potential for future regenerative medicine applications.

729. Engineered Phage Matrix Stiffness-Modulating Osteogenic Differentiation

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Although Biochemical cues in extracellular matrices plays a critical role in regulating cellular growth and faith, their physical cues as stiffness in guiding stem cells has not been well studied so far. Herein, we demonstrate an engineered phage mediated matrix for osteogenic differentiation with controlled stiffness by crosslinking the engineered phage displaying RGD and HPQ with various concentrations of streptavidin or polymer, PDDA. Osteogenic gene expressions showed that they were specifically increased when MC3T3 cells were cultured on the stiffer phage matrix than softer one. Our phage matrices can be easily functionalized using chemical/genetic engineering and used as a stem cell tissue matrix stiffness platform for modulating differential cell expansion and differentiation.

730. Integrating HDAd5/35++ Vectors as a New Platform for Hematopoietic Stem Cell Gene Therapy of Hemoglobinopathies

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Current hematopoietic stem cell (HSC) gene therapy for hemoglobinopathies involve self-inactivating lentivirus (SIN-LV)-based vectors expressing β-globin (either wild-type or anti-sickling variants of β-globin) or the fetal form, γ-globin. Because the vectors require an erythroid-specific promoter, SIN-LV vectors for globin gene therapy are relatively large and therefore difficult to produce at high titers. This, in turn, influences the cost for a gene therapy. Because of the structure and/or size of globin SIN-LV vectors, the HSC transduction frequency is relatively low. We propose integrating, CD46-targeted, high-capacity adenovirus HDAd5/35++ vectors as an alternative vector platform for HSC gene therapy. We generated an HDAd5/35++ vector with a 11.8kb transgene cassette containing a 5kb β-globin LCR/promoter version controlling the expression of a full-length human γ-globin gene as well as a EF1α-mgmt^{P140K} expression cassette which allows for drug-controlled increase of γ-globin expressing erythrocytes. Transgene integration from this vector is achieved by a hyperactive Sleeping Beauty (SB100x) transposase provided by a second HDAd5/35++ vector. In a first set of studies, we transduced bone marrow lineage-depleted cells from human CD46-transgenic mice and transplanted them into lethally irradiated recipients. The percentage of γ-globin positive cells in peripheral blood erythrocytes and erythroid cells in the bone marrow of primary and secondary transplant recipients was 90 to 100%. The γ-globin level was 10-20% of adult mouse globin. Transgene integration was random without a preference for genes. A second set of studies was performed with peripheral blood CD34$^+$ cells from mobilized healthy donors. Transduced human HSCs cells were transplanted into myeloablated NSG mice. Engrafted human HSCs were harvested from the bone marrow at week 10 after transplantation and differentiated *ex vivo* into erythroid cells. At the end of the differentiation process, we detected ectopic γ-globin expression at a level of 20% of adult α-globin. Our studies suggest that our HDAd35++ vector allows for efficient transduction of long-term repopulating HSCs and high-level, almost pancellular γ-globin expression in erythroid cells. The percentage of γ-globin expressing cells achieved in our study would be curative for β-thalassemia. Integrating HDAd5/35++ vectors have a number of potential advantages over SIN-LV vectors for globin gene therapy: *i)* The production of HD-Ad5/35++ vectors does not require large-scale plasmid transfection and yields high viral titers, which will greatly reduce the costs of clinical gene therapy. *ii)* HDAd5/35++ vectors do not require cell cycling for transduction. *iii)* They have an insert capacity of 30kb. *iv)* Integration is mediated by the SB100x transposase system, which functions independently of cellular factors. *v)* HDAd5/35++ vectors do not have a preference of transgene integration into active genes.

1004. Use of Oncolytic Adenovirus Expressing IFN Alpha as a Tool to Improve IFN-Based Chemoradiation Regimen to Treat Pancreatic Cancer

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Pancreatic Ductal Adenocarcinoma (PDAC) is the 3rd cause of cancer related death in United States. Aside from curative resection of tumors, there is not a highly effective therapy to treat PDAC. As patients are usually diagnosed with advanced stage of PDAC, more than 80% of them are not eligible to undergo surgery, resulting in post-diagnosis survival of 3-4 months, and 5-year survival of 6%. Despite the discouraging scenario, PDAC clinical trials using adjuvant therapy combined with IFN-α (IFN), 5-FU, CDDP, and radiation were improved the 2-year survival of patients by 20-41%, and the 5-year survival by 35%. In this sense, IFN-therapy is currently one of the few therapeutic regimens able to remarkably improve the short and long-term survival of patients. Despite high efficacy, drawbacks of therapy included high IFN systemic toxicity, which resulted in increased patient drop-out from trials, and low levels of IFN in tumors, which hampered IFN chemoradiosensitization capability in the therapy. Aiming to improve efficacy and overcome drawbacks of the IFN therapy, we studied the use of IFN-expressing replication competent oncolytic adenovirus (OAd) vectors in combination with chemoradiation mimicking the aforementioned IFN and chemotherapy-based clinical trials. As IFN-therapy can stimulate tumor specific immune response, we first evaluated effect of OAd vector expressing hamster IFN (OAd-hamIFN) in a syngeneic immunocompetent hamster model of PDAC. To complete our understanding of the interaction and efficacy of an IFN-expressing vector used in IFN-therapy, we later accessed effect of a human IFN-expressing OAd (OAd-IFN) in immunodeficient mice bearing human PDAC xenografts. In both studies, oncolytic adenovirus vectors included 5/3 fiber modification to increase infectivity of virus in PDAC cells. To improve OAd spreading and oncolytic effect in PDAC cells, and to achieve replication-dependent expression of IFN, Adenovirus Death Protein (ADP) and respective IFN genes were included in adenovirus (Ad) E3 region. To restrict replication of the human IFN expressing OAd (OAd-IFN) to human PDAC cells, the Cox-2 promoter was added upstream of Ad E1 region, the region responsible to initiate viral replication. Combinations of 5-FU, radiation, and 5-FU + radiation with OAd-hamIFN in hamster PDAC cells, or with OAd-IFN in human PDAC cells resulted in highly synergistic and cytotoxic combinations in vitro. Studies in syngeneic hamster PDAC model showed that inclusion of OAd-hamIFN in combination with therapeutics used in IFN-therapy improved treatment efficacy, and that use of the virus in treatment mimicking the IFN-therapy (OAd-hamIFN + 5-FU + Radiation) was the most effective therapy in the study. When analyzing effect of OAd-IFN in combination with 5-FU + CDDP in human PDAC cells, combinations were shown to be antagonistic and weakly cytotoxic. However, addition of radiation to treatment (OAd-IFN + (5-FU + CDDP) + radiation) overruled chemotherapy antagonism, resulting in highly synergistic and extremely potent treatments in vitro and in mice bearing PDAC xenografts. As radiation abolished antagonism of chemotherapy to

virus in vivo, we tested different radiation protocols in combination with OAd-IFN. We concluded that administration of radiation before infection of tumors with OAd-IFN improved treatment efficacy, and suggested that radiation protocol should be further optimized in combinations representing IFN-therapy. In summary, our data strongly supported inclusion of an IFN expressing OAd in treatments mimicking IFN-therapy. As this therapy is one of the few therapeutic regimens proven to improve the short and long-term survival of PDAC patients, development of a virus based IFN-therapy might result in an impactful treatment against PDAC.

Cancer - Immunotherapy, Cancer Vaccines III

731. Disruption of the AHR Signaling Pathway Enhances IL-17 Production but Negatively Impacts Proliferation of Transgenic TCR and CAR T Cells

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Dysregulated tryptophan metabolism has been implicated in the pathogenesis of multiple cancers. Elevated activity of enzymes such as indoleamine 2,3 dioxygenase (IDO1) leads to enhanced degradation of tryptophan to immunosuppressive metabolites such as kynurenine (kyn). In T cells, kynurenine activates an immunosuppressive transcriptional program by binding to the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor. Subsequent AHR translocation to the nucleus drives a transcriptional cascade resulting in decreased cytokine production and T cell proliferation. Pharmacological modulation of tryptophan metabolism has produced promising clinical outcomes in several oncology areas. Additionally, previous studies have demonstrated decreased activity of chimeric antigen receptor (CAR) T cells against cell lines with high IDO1 activity, suggesting that interfering with tryptophan metabolism may also enhance CAR T cell function. As an alternative strategy to pharmacological inhibition which acts on the tryptophan degradation pathway in tumor cells, here we describe megaTAL editing at the AHR gene in T cells in an effort to render them resistant to kyn-mediated immunosuppression. Electroporation of activated T cells with mRNA encoding an AHR targeting megaTAL produced more than 80% gene editing rates as measured by sequencing, while flow cytometry analysis of gene-edited T cells showed substantial reduction in AHR protein expression. Following activation, megaTAL-treated T cells show reduced upregulation of the AHR target gene *Cyp1A1* as measured by qPCR, indicating that gene editing decreases AHR transcriptional activity. We used two different model antigens to determine the functional consequence of AHR deletion on T cell function. T cells were transduced with lentiviral vectors encoding either a TCR specific for the cancer testis antigen NY-ESO-1 or a CAR specific for the Epidermal Growth Factor Receptor (EGFR). In short-term tumor co-culture assays, the AHR-edited NY-ESO-1 and EGFR CAR T cells exhibited similar cytolytic activity and cytokine production when compared to non-edited controls. Notably, AHR editing resulted in increased IL-17A cytokine production from both CAR and TCR T cells, consistent with a shift towards a Th17 phenotype. However, despite similar levels of viability, AHR-edited T cells demonstrated decreased proliferation and cytokine production following repeat antigen exposure. Combined, our findings confirm that AHR signaling negatively regulates IL-17 cytokine production and describe a previously unappreciated role for AHR in promoting T cell expansion.

732. Flipping the Script on the Tumor Microenvironment: A Novel Signal Conversion Platform That Exploits Tumor-Derived TGF-β to Enhance CAR T Cell Effector Function

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Numerous immune-suppressive mechanisms exist within the tumor microenvironment that may hinder the efficacy of adoptively transferred T cells. One such mechanism is mediated by TGF-β, a cytokine secreted by tumor cells and infiltrating suppressive immune cells that directly inhibits effector T cell activity. Effector T cells express the TGF-β receptors TGFBR1 and TGFBR2, and exposure of T cells to TGF-β induces hetero-dimerization of these receptors and phosphorylation of the major TGF-β signal mediators SMAD2 and SMAD3. Phosphorylated SMAD proteins (pSMADs) induce a suppressive transcriptional program that ultimately leads to reduced cytokine production, reduced cytotoxicity, and a failure to proliferate in response to antigen stimulation. In several previous reports, a dominant negative receptor (DNR) version of TGFBR2, that does not contain signaling domains, was shown to protect T cells from the impacts of TGF-β by blocking the ability of TGF-β to induce pSMADs. We have been developing a novel TGF-β signal conversion platform that provides a T cell supportive signal upon exposure to TGF-β. This platform utilizes co-expression of chimeric variants of TGFBR2 and TGFBR1 where the TGF-β-binding domain of each receptor is fused to the transmembrane and intracellular signaling domains of a T cell simulating interleukin receptor. Based on the ability of lentiviral vectors to express large and complex gene expression cassettes, we constructed vectors encoding both chimeric TGF-β receptors (CTBR) in the context of either a CAR or transgenic TCR, which in the case of the CTBR/TCR vector, must express four unique proteins from a single vector. Here, we report the in-depth characterization of the functional attributes of CTBR co-expressing CAR T cells, including gene expression, multiplexed cytokine production, cytotoxicity, survival, and metabolic function. As anticipated, the STAT signaling signature induced by a particular CTBR was predictive of the functional consequence of exposure to TGF-β. In the case of a CTBR based on IL-12 signaling domains (CTBR12) that strongly induced STAT4 phosphorylation, CTBR12 expressing T cells secreted significantly greater amounts of IFNγ than control T cells following activation in the presence of TGF-β. In the case of a CTBR based on IL-7 signaling domains (CTBR7) that strongly induced STAT5 phosphorylation, CTBR7 expressing T cells exhibited prolonged survival in the absence of exogenous IL-2 support

but presence of TGF-β. Next, we evaluated CTBR expressing T cells in a solid tumor driven serial re-stimulation assay in the presence of TGF-β. Antigen driven T cell expansion was severely limited by exposure to TGF-β, and either CAR or transgenic TCR expressing cells ultimately lost cytotoxic activity over time. Importantly, in this suppressive setting CTBR expressing cells maintained their ability to expand, produce cytokine and kill tumor targets in the presence of TGF-β. Experiments to address the effectiveness of this approach in solid tumor xenograft models are in progress. Together, these data demonstrate the successful development of a TGF-β signal conversion platform that transforms the inhibitory effects of TGF-β exposure into a STAT signal that supports T cell effector function. The specific impact on T cell function is dependent on the nature of the signaling domains utilized. This platform has demonstrated the desired functional outcomes in the context of both CAR T cells and transgenic TCR T cells, and has the potential to produce superior T cell responses in the immunosuppressive tumor microenvironment.

733. Transcriptional Signatures of CD28-ζ and 4-1BB-ζ Containing CAR T Cells

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Background Chimeric antigen receptors (CARs) targeting CD19 have produced impressive outcomes for the treatment of B cell malignancies. Using RNA sequencing, we determined the transcriptional changes downstream of CARs with different co-stimulation domains pre- and post-antigen stimulation. **Methods** We isolated, transduced and expanded T cells that expressed one of 4 CAR constructs (ζ, 28ζ, 4-1BBζ and delζ). All CARs contained identical CD19-directed scFv and CD8 hinge and transmembrane domains. CAR T cells were rested for a week and then stimulated for 4 or 24 hours through their CAR using irradiated $CD19^+$ NALM6 leukemia cells or through their TCR using anti-CD3/CD28 beads. CAR+ T cells were then sorted into $CD4^+$ or $CD8^+$ and bulk RNA sequencing was performed using Smart-Seq2 protocol. 5 different stimulation time points/conditions were used for 3 human donors with technical duplicates resulting in over 240 sequenced samples for analysis. Paired-end reads were aligned and quantified and differential gene expression calculated using DESeq2. Significant differentially expressed (DE) genes had a FDR cutoff of <0.1. **Results** We interrogated the differences between the CAR T cells at rest (time 0) or after activation through their CAR. Interestingly, many of the DE genes were significant before stimulation in all the functional CARs compared with delζ. We defined these DE genes common in comparisons with delζ to all functional CARs as the signature for tonic signaling from the CD3ζ chain. Furthermore, we identified a set of genes that is DE between 4-1BBζ and CD28ζ CAR T cells at all time points, many of which are involved in cytokine and other immune signaling pathways. We found that many of the upregulated genes in 4-1BBζ CARs were HLA class II genes and we validated this increased expression of HLA-DR at the protein level by flow cytometry. GSEA analysis showed an increase in TNF and IFNγ signaling in 4-1BBζ CARs compared to CD28ζ 24 hours after antigen stimulation. There were also many DE cytokine and cytokine receptor genes 24 hours after antigen stimulation between 4-1BBζ and CD28ζ CARs. These included IL21, IL21R, IL12RB2 and IL23R which were all upregulated in 4-1BBζ CARs. CD28ζ CARs had significantly increased PD-1 and anti-apoptotic gene expression compared to 4-1BBζ CARs. Across all time points there was an enrichment for human T_H1 signature genes in the 41BBζ compared to the CD28ζ CD4+ CAR T cells (p-value<0.0005). **Conclusions** We identified a signature for tonic signaling which indicated that the mere expression of the CAR bearing a CD3ζ chain resulted in a specific transcriptional response. We found many of these DE genes between CD28ζ and 4-1BBζ CARs are involved in class II antigen presentation, cytokine signaling, metabolism, apoptosis and other immune pathways. From this data, we concluded that the type of co-stimulation domain plays an early role in the polarization of T helper subsets which could affect the quality of help provided to CD8+ T cells as well as their direct anti-tumor potential.

734. CCR2 Modification Improves Trafficking of Chimeric Antigen Receptor T Cells to Glioblastoma in Preclinical Model of Disease

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Malignant gliomas (MG) are the most common and difficult-to-treat brain tumors. The outstanding efficacy of chimeric antigen receptor (CAR)-modified T cells against hematological malignancies gives hope that they can be programmed to target solid tumors like MG. We have recently demonstrated that the local intratumoral delivery of CAR.CD28.ζ T cells targeting tumor-associated antigen interleukin-13 receptor alpha 2 (IL13Rα2) improves the survival of glioma-bearing mice. In this study, we demonstrate that only a small fraction of CAR T cells reaches glioma after systemic injection. In order to circumvent the inefficient trafficking of CAR T cells from the periphery directly to glioma tissue, we took advantage of the increased expression CCL2, a chemokine secreted by the glioma microenvironment, and have modified our IL13Rα2-CAR.CD28.ζ T cells to overexpress CCR2, a receptor for CCL2 (IL13Rα2-CAR.CD28.ζ.CCR2). Truncated constructs, IL13Rα2-CAR.Δ and IL13Rα2-CAR.Δ.CCR2, which lack intracellular signaling domains, served as controls. A robust expression of CCR2 was detected on the cell surface of IL13Rα2-CAR.CD28.ζ.CCR2 and IL13Rα2-CAR.Δ.CCR2 T cells. Flow cytometric analysis determined that transgenic expression of CCR2 in IL13Rα2-CAR.CD28.ζ T cells improved their infiltration to the brain parenchyma of glioma-bearing mice. IL13Rα2-CAR.CD28.ζ.CCR2 infiltration was further improved if glioma-bearing mice underwent lymphodepletion prior to their systemic delivery. Collectively, our data demonstrate that the transgenic expression of CCR2 in CAR T cells improves their trafficking to the glioma site and should be further investigated as a viable therapeutic option for patients with MG.

735. Quantitative Control of Gene-Engineered T Cell Activity through Covalent Attachment of Targeting Ligands to a Universal Immune Receptor

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CAR T cells have shown great success in the treatment of CD19+ hematological malignancies, which has lead to their recent approval by the FDA as treatment modality. However, their broad use is limited since a CAR targets a single tumor associated antigen (TAA), which is not effective against tumors with heterogeneous TAA expression or emerging antigen loss variants. Further, stably engineered CAR T cells can continually proliferate and activate in response to antigen, potentially causing fatal toxicity without a method of control or elimination. To overcome these issues, we and others have developed various universal immune receptors (UIRs) that allow for targeting of multiple TAAs by T cells expressing a single receptor. These UIRs are a promising new technology, but their reliance on *noncovalent* interactions between receptor and targeting ligand can lead to potential issues of suboptimal affinity, specificity and activity. We have developed a UIR platform that employs SpyCatcher and SpyTag proteins which, when combined, form a covalent bond with high efficiency both *in vitro* and *in vivo*. The SpyCatcher immune receptor is composed of an extracellular SpyCatcher domain attached to intracellular T cell signaling motifs. To confer and control redirected specificity to SpyCatcher-expressing T (Spy-T) cells, TAA-specific antibodies labeled with SpyTag are covalently bound to the receptor post-translationally. Here, we show that human T cells can efficiently express the SpyCatcher immune receptor. This allows for the quantitative and temporal control of T cell activation, cytokine secretion, and tumor cell lysis with the addition of SpyTag-labeled targeting antibody. The adaptability of Spy-T cells allowed them to recognize an array of different antigens, thus broadening the response against tumors with heterogeneous TAA expression. Additionally, dose-dependent arming of Spy-T cells with SpyTag-labeled targeting antibodies allowed for controlled detection of high vs low level antigen expression, as an additional means of safety. Finally, the ability to post-translationally modify these receptors has allowed for the monitoring of receptor turnover kinetics both in the absence and presence of antigen-expressing tumor cells. In conclusion, the SpyCatcher immune receptor is the first UIR designed for post-translational *covalent* attachment of targeting ligands that permits timed "on demand" redirection of T cells against a diverse array of antigens, addressing limitations of safety and antigen diversity by conventional CAR T cell therapy.

736. Abstract Withdrawn

737. Generation of Human Memory Stem T Cells Redirected against Tumor Antigens and Resistant to Inhibitory Signals by Genome Editing

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Introduction Cancer cells exploit inhibitory receptors (IRs) expressed by T cells for protection from immune attack. Resulting tumor-specific T cells exhibit an unresponsive functional profile named as T cell exhaustion. Tim3 and LAG3 represent highly expressed inhibitory receptors in exhausted T cells infiltrating solid tumors. Furthermore, we recently showed that a large fraction of BM infiltrating $CD8^+$ cells co-express inhibitory receptors, including Tim3, in patients with acute myeloid leukemia relapsing after allogeneic hematopoietic stem cell transplantation. These observations advocate for strategies to overcome inhibitory pathways in the context of adoptive cell therapy with genetically engineered lymphocytes. **Purpose** We aim to simultaneously redirect T cell specificity and permanently disrupt inhibitory receptors by CRISPR/Cas9 in memory stem T cells (T_{SCM}) to be used in adoptive T cell therapy. **Methods** We designed and tested multiple gRNAs targeting Tim3, LAG3 and the TCR α chain constant region (TRAC) loci in K562-iCas9 cell line and primary T cells. Primary T cells were stimulated with beads anti-CD3/CD28+IL7/IL15 and electroporated with Cas9/gRNA ribonucleoproteins (RNPs). The frequency of NHEJ was assessed with FACS analysis, surveyor assay and ddPCR. A lentiviral vector (LV) encoding for an NY-ESO1-specific TCR (LV-NYESO1-TCR) was used for TCR gene editing. **Results** Once the gRNAs with the highest cut efficiency were identified in a K562-iCas9 cell line, stimulated primary human T cells were electroporated with RNPs targeting Tim-3, LAG-3 or TRAC genes. Phenotypic and genomic assessment at day +4 showed high level of inhibitory receptor gene disruption, with up to 60-70% at the Tim3 locus and 80-90% at the LAG3 site. We obtained up to 98% of $CD3^{neg}$ cells after TRAC gene disruption. $CD3^{neg}$ cells were efficiently transduced (70-85%) with LV-NYESO1-TCR. The TCR gene editing protocol produced a large number of edited T_{SCM} cells that proved specific and effective in killing $NY\text{-}ESO1^+$ multiple myeloma cells. Most importantly, we then combined Tim3 disruption with TCR gene editing in a single protocol, and we obtained approximately 47% of $Tim3^{neg}$ and TCR redirected T cells. More than 90% of edited cells showed a T_{SCM} functional phenotype and proved effective and specific in killing $NY\text{-}ESO1^+$ tumor targets. **Discussion** By exploiting the plasticity and multiplexity of CRISPR/Cas9 we generated innovative adoptive T cells therapy products, directed against a selected tumor antigen, and resistant to inhibitory signals. The final goal of our approach is the adoptive transfer of tumor-specific long-living memory stem T cells resistant to inhibitory signals to cancer patients.

738. Engineered Extracellular Vesicles Carrying Heterodimeric IL-15: Production, Characterization and Tumor Delivery

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BACKGROUND: Interleukin-15 (IL-15) is an immunostimulatory cytokine that leads to proliferation and activation of NK and cytotoxic T cells. In humans, non-human primates and mice it is found primarily as a cell-associated or secreted heterodimer consisting of IL-15 non-covalently bound to a stabilizing polypeptide named IL-15 Receptor alpha. Heterodimeric IL-15 (hetIL-15) is being tested clinically for treatment of cancer. We sought to develop technologies that would allow for translational development of extracellular vesicles (EV) as versatile, targetable, immunotherapy vectors enriched in hetIL-15. EV are liposome-like vesicles secreted by eukaryotic cells, which mediate biological effects through protein and nucleic acid cargo. **METHODS:** DNA constructs encoding novel forms of IL-15 were generated, expressed in HEK293 cells, and optimized with respect to protein expression level, EV-enrichment, and *in vitro* bioactivity. EV were purified from cell culture media of HEK293 cells grown in large conventional flask cultures or from hollow-fiber bioreactor cultures. Purification of EV using scalable cGMP-compatible methodologies (sequential tangential flow-filtration [TFF] and size-exclusion chromatography [SEC]) were tested with respect to EV yield, purity, and maintenance of EV-associated cytokine. *In vitro* cellular uptake of fluorescently labelled EV was studied in the presence of selective uptake inhibitors to elucidate uptake mechanisms. *In vivo* biodistribution of EV was tested in mice with subcutaneously implanted 4T1 breast cancer tumors to address feasibility of tumor targeting. **RESULTS:** EV purified from cells expressing the native form of hetIL-15 (IL-15/IL-15 receptor alpha) incorporate the cytokine in a functional form. Cells expressing an optimized human IL-15/IL-15 receptor alpha-Lactadherin fusion protein produced EV carrying significantly greater amount of bioactive cytokine. The mouse homologue of Lactadherin was previously shown to facilitate EV loading via interaction with phosphatidylserine on the vesicular membrane. Hollow-fiber bioreactor cell culture yielded significantly more EV per unit volume, in a chemically-defined medium that facilitated downstream purification. Sequential TFF+SEC allowed for rapid purification of EV, with separation of IL-15-enriched vesicles from non-EV entities, including large protein complexes. Mass spectrometry and Gene-set enrichment analysis of purified EV-associated proteins showed significant enrichment of vesicle proteins over other purification methods. *In vitro* cellular uptake of EV was mediated by multiple scavenger receptors, but also occurred via non-receptor mediated mechanisms, depending on the cell type. *In vivo*, EV primarily targeted liver, lung, and spleen; blockade of Class A Scavenger Receptors decreased liver uptake and significantly enriched tumor targeting of EV. **CONCLUSIONS:** EV comprise a promising vector for delivery of multiple therapeutic molecules, including immunostimulatory proteins and nucleic acids. We piloted a development pipeline for engineering, production, and delivery of EV carrying hetIL-15 that may also be applicable for customized EV from other cell sources with diverse therapeutic properties. Further research in controlling the biodistribution of EV is necessary to maximize therapeutic potential in cancer and other diseases.

739. Engineering Car T-Cells with an Integrated off Switch to Enhance Safety Performance

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The last years have seen the adoptive transfer of engineered T-cell as a key player in the development of new treatments against cancer. The high remission rates observed in clinical trials are however mitigated by potentially life-threatening side effects potentially due to CART-cells attacking healthy tissues, with low levels of targeted tumor-associated antigens (on-target, off-tumor effects). Therefore, endowing T-cells with a therapeutically relevant CAR could be a challenging process as few true tumor specific antigens have been identified. In order to work around this bottleneck and to improve the overall safety of these therapeutic approaches, several strategies including combinatorial targeting and suicide switches have been developed. The possibility to spatio-temporally regulate CAR T-cell functions in a non-lethal fashion using small molecules could allow better mitigating potential toxicity, improving the overall therapeutic outcome. Here, we developed a single component system to control CAR T-cell cytolytic properties with a small molecule drug in a switch OFF fashion. We utilized an approach combining a protease and a degron to control CAR stability in primary T-cells via a protease inhibitor. The addition of the Asunaprevir (ASN) protease inhibitor led to the selective and dose-dependent, decrease of CAR surface presentation and CAR T-cell cytolytic properties. We foresee that such a CAR control approach would benefit to the safety of clinical applications and the *ex vivo* production of CAR T-cells.

740. Development of a Novel Synthetic Consensus DNA Vaccine That Targets Multiple MAGE-A Family Members for Anti-Cancer Immune Therapy

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Cancer/testis (CT) antigens have emerged as attractive targets for cancer immune therapy due to their over-expression in tumor tissues and lack of expression in normal tissues. In particular, several clinical studies have been initiated to target the MAGE-A family of CT antigens for melanoma. These have included recombinant protein immunization

and TCR based gene therapy for MAGE-A3. The recombinant protein immunizations resulted in poor CD8+ T cell responses and lack of efficacy thus far in the clinic. TCR based gene therapy induced robust immune responses but unexpected toxicity. There is therefore a need to develop safe and effective therapies targeting the MAGE-A family of proteins for cancer therapy. In this study we performed a thorough analysis of MAGE-A RNA expression in The Cancer Genome Atlas (TCGA) and demonstrated that a high proportion of patients, in particular patients with melanoma and lung squamous cell carcinoma, exhibit expression of multiple MAGE-A family members simultaneously within the same tumor sample. Based on this information, we designed a consensus MAGE-A DNA vaccine that retains high homology (>85%) to multiple MAGE-A isoforms. Upon delivery of the mouse consensus MAGE-A vaccine intramuscularly followed by electroporation (EP) in C57Bl/6 mice, we detected robust IFN-γ and TNF-α CD8+ T cell responses against multiple MAGE-A isoforms, including MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A5, MAGE-A6 and MAGE-A8 ($p<0.005$). Furthermore, we detected robust cytotoxic CD107a/IFN-γ/T-bet triple-positive CD8+ T cells in mice immunized with the MAGE-A vaccine. We evaluated the potency and cross-reactivity of this MAGE-A DNA vaccine in genetically diverse, outbred mice. We found that the majority of these mice (14/15) were capable of mounting a cross-reactive immune response and breaking tolerance to multiple MAGE-A isoforms simultaneously. We tested the anti-tumor activity of this MAGE-A DNA vaccine in *Tyr::CreER;BRAF*$^{Ca/+}$*;Pten*$^{lox/lox}$ transgenic mice that develop melanoma upon application of tamoxifen on the skin. The MAGE-A DNA vaccine, when delivered therapeutically, was able to significantly slow tumor growth in this autochthonous mouse model and improve median mouse survival by 2-fold (50 days, $p<0.005$) compared to control mice. These results support the use of optimized MAGE-A consensus vaccines for cancer immune therapy.

741. Inactivated Sendai Virus Particles Upregulate Cancer Cell ICAM-1 Expression with Enhancing NK Cell Sensitivity on Cancer Cell

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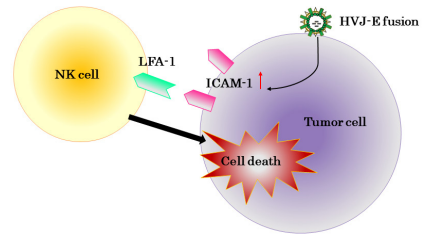
In this study, we found that inactivated Sendai virus (hemagglutinating virus of Japan; HVJ) envelope (HVJ-E) induced the production of intercellular adhesion molecule-1 (ICAM-1, CD54), a ligand of LFA-1, in several cancer cell lines through the activation of NF-κB downstream of retinoic acid-inducible gene I (RIG-I) and the mitochondrial antiviral signaling (MAVS) pathway. The upregulation of ICAM-1 on the surface of cancer cells increased the sensitivity of cancer cells to NK cells. In general, ICAM-1 is a transmembrane glycoprotein that is induced by retinoic acid, virus infection, and cytokines such as IL-1-β, TNF-α, and IFN-γ. We have already reported that HVJ-E has multiple anti-cancer effects, including induction of cancer-selective cell death and activation of anti-cancer immunity. HVJ-E stimulates DCs to produce cytokines and chemokines such as IFN-γ, IL-6, CCL5 and CXCL10, which activate both CD8+ T cells and NK cells and recruit them to the tumor microenvironment. However, the effect of HVJ-E on modulating the sensitivity of cancer cells to immune cell attack has yet to be investigated. To investigate changes in NK cell ligands in cancer cells induced by HVJ-E, we measured RNA expression levels of a number of NK cell ligands in MDA-MB-231 (a human breast cancer) and PC3 (a human prostate cancer) cells by quantitative real-time PCR. RNA expression levels of ICAM-1 mRNA was significantly increased in both cell lines stimulated with HVJ-E for 24 hours. Expression of ICAM-1 on the cell surface was confirmed by flow cytometry analysis, and the ICAM-1 expression on the cancer cell surface was increased with HVJ-E treatment compared with that in non-stimulated cells. Moreover, in the non-cancerous normal cells, HVJ-E failed to upregulate the expression of ICAM-1. Our previous study has identified that HVJ-E RNA fragments are able to be recognized by RIG-I/MAVS. To further confirm whether HVJ-E-induced ICAM-1 overexpression is dependent on the RIG-I/MAVS system, we knocked down the RIG-I or MAVS gene in MDA-MB-231 cells and treated the cells with HVJ-E. HVJ-E-induced ICAM-1 expression was reduced in cells transfected with either RIG-I or MAVS siRNA. Then, MDA-MB-231 cells were inoculated to SCID mice and treated them with HVJ-E or PBS. The tumor growth was significantly inhibited by HVJ-E treatment. Furthermore, to determine if the anti-tumor effect of HVJ-E in the MDA-MB-231 tumor model is related to NK cells, the NK cells in mice were depleted with the anti-asialo-GM1 antibody. As a result, HVJ-E-induced tumor suppression was attenuated by NK cell depletion. Next, we sought to demonstrate whether a deficiency of ICAM-1 abolishes the HVJ-E-induced enhancement of NK cell cytotoxicity to cancer cells. ICAM-1 knockout in MDA-MB-231 cells using the CRISPR/Cas9 significantly reduced the killing effect of NK cells on ICAM-1-depleted MDA-MB-231 cells, *in vitro*. In conclusion, these findings suggest that HVJ-E enhances the NK cell sensitivity of cancer cells by increasing ICAM-1 expression on the cell surface, which results in the promotion of NK cell anti-cancer cytotoxicity. This study identified a novel mechanism underlying HVJ-E anti-tumor activity. HVJ-E can increase the sensitivity of cancers to immunotherapy by modifying the gene expression pattern in cancer cells.



742. Pre-Clinical Experiments of Cart Cells Identifying TSHR as a Potential Target against Metastatic Thyroid Cancer

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Background: Despite mature and effective treatment for general thyroid cancer, there is a major unmet medical need for patients with advanced or metastatic thyroid cancer, including papillary thyroid (PTC), thyroid follicular carcinoma (FTC), as well as patients unable to carry out surgery. Further research with novel treatments are

warranted. Thyroid stimulating hormone Receptor (TSHR) is secreted by the pituitary, interacting with Thyroid stimulating hormone (TSH). TSHR is expressed in the mammary normal thyroid tissue as well as gland carcinomas. We screened the databases and identified TSHR as a potential CART target. **Methods:** Anti-TSHR CART cells were constructed based on 41BB co-stimulation domain transduced by lentivirus. TSHR CART cells were co-cultured with TSHR positive cell line in vitro and tested by comparing the killing efficacy of TSHR positive cell line versus TSHR negative cell line. In addition, we built thyroid cancer cell transplanted immune deficient mice model to evaluate CAR-T safety and efficiency in vivo. **Results:** TSHR positive cell line co-cultured with CAR-T cells were killed significantly, while the one co-cultured with none transduced T cells remained alive with significant difference. IFN-r release increased as E/T ratio escalation in the co-culture experiment. In vitro killing experiment show potent anti-tumor activities of TSHR CART cells. In the animal model, CART cells inhibit the growth of cancer cells. **Conclusion:** Our pre-clinical experiments showed potent efficacy and specificity of TSHR CART cells, suggesting TSHR as a very promising potential target for CART cell therapy against metastatic thyroid cancer. Further clinical trials are necessary to prove its safety and efficacy in the patients with metastatic thyroid cancer.

743. Chimeric Antigen Receptor T Cells Redirecting EPHB4 for the Treatment of Human Rhabdomyosarcoma

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Background The survival rate of rhabdomyosarcoma (RMS) is dismal. The EPHB4 receptor, which is associated with the malignant phenotype of various tumors, is strongly expressed in RMS, but at extremely low levels in normal tissue. In this study, we developed novel chimeric antigen receptor (CAR)-T cells that can target EPHB4 and kill RMS cells. **Material and methods** We generated a *piggyBac* transposon-mediated CAR-T cells, which enabled transduced cells to recognize EPHB4-positive RMS cells. The positivity of CAR transgene and the phenotype of transduced cells were analyzed by flow cytometry. To evaluate the cytotoxic activity, the transduced cells were co-cultured with an RMS cell line, Rh30, and the transduced cells were injected via tail vein of RMS xenograft-bearing mice. **Results** We successfully generated EPHB4-CAR-T cells, with a positivity of 27.95±3.85% for EPHB4-CAR at day 14 after transduction. Immunophenotyping of transduced cells revealed that 95.92±1.78% were positive for CD3, 13.20±1.10% were positive for CD4, and 86.79±1.10% were positive for CD8. Notably, EPHB4-CAR-T cells exhibited CD8+/ CD45RA+/ CCR7+ fraction which was closely related to naïve/memory stem cell phenotype, indicating their proliferative potential in response to antigen stimulation. When co-cultured with Rh30 cells, EPHB4-CAR-T cells produced IFNγ more abundantly than CD19-CAR-T cells (the concentration of IFNγ; 25754.77±935.65pg/ml in EPHB4-CAR-T cells and 63.94±10.70pg/ml in CD19-CAR-T cells, respectively), and EPHB4-CAR-T cells effectively eliminated Rh30 cells, with <1% of Rh30 cells remaining after 72-h co-culture. Furthermore, EPHB4-CAR-T cells could debulk the RMS tumor in vivo. **Conclusion** We have demonstrated that EPHB4-CAR-T cells effectively eliminate RMS cells. The EPHB4-CAR-T cells may therefore be used as a novel adoptive T-cell therapy for RMS.

744. Glycosurfaceome Mapping in Multiple Myeloma for the Elucidation of Novel Diagnostic and Immunotherapy Targets

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Multiple myeloma (MM) is a hematological malignancy responsible for 2.1% of all cancer deaths in the USA. The development of novel therapies over the past decade, such as proteasome inhibitors and immunomodulatory drugs, has improved MM patient outcomes and survival. That said, MM remains an incurable disease with a 5-year survival rate below 50%. Emerging immunotherapy strategies for the treatment of MM, such as antibody therapies and chimeric antigen receptor (CAR) gene therapies, may offer more durable and long-term clinical responses. To date, only a few antigens and pathways in MM have been pursued. The identification and characterization of novel MM antigens would provide new immunotherapy targets and new insights into MM biology. Approximately 70% of proteins on the surface of cells are glycosylated. We have applied a mass spectrometry-based proteomics approach in order to map the glycosurfaceome of MM. In the discovery phase of this approach, the glycosurfaceome of four MM cell lines was probed using cell surface capture technology. Multiple independent analyses were performed on 4 replicates of 100M cells for each line. Glycosylated surface proteins were labelled, captured, and analyzed by high resolution mass spectrometry. A total of 545 glycosylated surface proteins were identified on the MM cell lines. 97 glycoproteins were common between all the cell lines while 221 glycoproteins were unique to each line. Data from a B cell line and historical data from a wide range of cell lines and primary samples were used to subtract hits associated with expression on normal cells. Protein and RNA expression data from publicly available databases was also applied to this analysis, narrowing the focus of our study to 135 glycoproteins of interest. The second phase of this study involves a targeted assessment of glycoprotein expression on the surface of MM patient samples. Evaluation of the 135 glycoproteins of interest will be carried out by parallel-reaction monitoring (PRM)-based mass spectrometry using plasma cells isolated from multiple MM patient bone marrow samples. Linked patient data will be used to identify any proteins associated with outcomes such as survival and response to therapy. Next, glycoproteins found on MM samples, but with restricted expression on normal tissues, will be overexpressed using a mammalian system, purified, and panned by us against a phage library in order to identify novel single-chain antibodies. With these antibody reagents in hand, further validation of these targets on MM and normal

tissues will be carried out. Furthermore, antibody and CAR therapies - using our existing lentiviral vector backbone - will be pursued. Development of new methods for monitoring and treating MM continues to be an active area of research. Our approach allows the elucidation of new MM targets, which may be useful for diagnostic and therapeutic applications, ultimately leading to advancements in patient survival and quality of life.

745. Automated CAR T Production on CliniMACS Prodigy® in Serum-Free Cultures Supplemented with Il-2 or Il-7/Il-15

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Chimeric antigen receptor (CAR T) cell immunotherapy has shown great promise in the treatment of hematologic malignancies. Production of CAR T cells *ex vivo* typically involves T cell activation via CD3 and CD28, transduction with a lentiviral vector (LV) encoding the CAR molecule, and culture expansion in the presence of T cell homeostatic cytokines. The phenotype of the resulting CAR T product can have a great impact on the clinical outcome, and specifically, central memory T cells (T_{CM}) yield CAR T cells of superior functionality. We hypothesized that culture supplementation with IL-7/IL-15 in the CliniMACS Prodigy® automated CAR T production system would generate a better CAR T product as compared to supplementation with IL-2. We first generated anti CD19 CAR T cells on a small scale under serum-free conditions using varying concentrations of IL-2 and IL-7/IL-15 and CliniMACS TransAct® (anti-CD3/CD28) reagent for activation. LV produced via chemically-defined, serum-free process was utilized. Culture viability, expansion rate, T cell activation, CAR expression, CAR cytotoxic function and cytokine response were evaluated. We then translated select culture regimens to CAR T production in CliniMACS Prodigy®. We demonstrate that in small scale, reduction of the concentration of IL-15 (in the presence of IL-7) or IL-2 (alone), which share a common receptor β-chain, correlated with increased cell viability and expansion, accelerated differentiation into T_{CM}, and a shortened period of cellular activation as indicated by CD25/CD69 expression, but had no impact on CAR expression. All CAR T products exhibited specific killing and cytokine response against $CD19^+$ tumor lines Raji and Reh, but not against the $CD19^-$ line 293T, thus demonstrating specific cytotoxicity. Of note, by using lower IL-2 or IL-15 concentrations we minimized non-specific T cell cytotoxicity and inflammatory cytokine production seen in some donors against a $CD19^-$ $K562_{sensitive}$ cell line, without sacrificing antigen-specific CAR function. Surprisingly, CAR T cells generated in Prodigy using either high IL-2 or high IL-7/IL-15 cytokine regimens were similar, with higher T_{CM} content, greater expansion rate, and comparable CAR T function and viability *vs* CAR T cells produced in small scale under same regimens. Importantly, we did not observe non-specific responses against $K562_{sensitive}$ targets in Prodigy-generated CAR T products. Collectively, serum-free, automated CAR-T production in CliniMACS Prodigy® reduces the impact of cytokine regimens, while preserving favorable CAR T product characteristics, including high viability and expansion, prevalent T_{CM} phenotype, tumor-specific cytokine response, cytotoxic function, and reduced potential for non-specific reactivity.

746. CAR-NK Cells for Immunotherapy of Solid Tumors: Mediating Effective Non-Viral Genetic Engineering of NKG2D-Reprogrammed NK Cells

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Natural killer (NK) cells are being increasingly considered as an effective approach to cancer immunotherapy. The introduction of chimeric antigen receptors (CARs) into NK cells could potentially increase their killing ability. To generate safe and efficient CAR-expressing NK cells, it is necessary to develop non-viral carriers with good biocompatibility which can load and deliver genetic material effectively, while mediating NK cells' inherent resistance to exogenous gene uptake. Our goal is to develop efficient non-viral reprogramming approaches while inhibiting NK cells' gene uptake resistance mechanisms to generate safe CAR-redirected NK cells. **To achieve that**, we are developing a non-viral platform based on poly(β-amino ester) (PBAE) to deliver piggyBac-expressing CAR genes to NK-92 cells. PBAE was synthesized in a two-step reaction. Firstly, acrylate-terminated base polymers were prepared by Michael addition. Then, the piperazine-capped base polymers were further prepared by adding amine-containing small molecules. The cytotoxicity of PBAE on NK-92 cells was investigated through both CCK-8 and 7-AAD assays. The DNA-binding capability of PBAE was examined electrophoretically. We investigated the effect of BX795, an inhibitor of the TBK1/IKKε complex for its ability to enhance CAR uptake by NK cells. Transgene expression was evaluated by flow cytometry. To enhance NK-mediated cytotoxicity, we constructed a CAR gene targeting NKG2D (NKG2D-DAP10-CD3ζ) using the piggyBac transposon system. **We have found** that PBAE shows no obvious toxicity to NK-92 cells at concentrations as high as 80 μg/ml, and can load plasmid DNAs containing a GFP reporter gene (pEGFP-N2) efficiently; while inducing GFP expression with a 7-fold increase in mean fluorescence intensity compared to the untreated group. The use of BX795 enhances gene delivery but induces toxicity to NK cells. We are currently evaluating expression function of our NKG2D-DAP10-CD3ζ sequence alongside a hyperactive form of the transposase (iPB7) gene, both of which showed high-binding affinity toward PBAE. We are also evaluating the cytotoxic activity against a range of solid tumors, including skin melanoma and non-small cell lung cancer. **By showing** that non-viral vectors containing different CAR genes could be efficiently constructed using the piggyBac transposon platform; we have demonstrated a path toward safer, non-viral engineering of CAR-NK cells for immunotherapies of solid tumors.

747. *Minicircle* Goes Viral - an Overview

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Tomorrow is now - at least for non-viral DNA molecules used for gene- or cell therapy, vaccination or manufacturing viral vectors. Recently,

the vision of a "plasmid" that is free of any selection markers, CpG containing backbone structures and even the origin of replication became reality: Minicircle (MC). MC have proven to be a reliable tool for efficient transgene expression in eukaryotic cells *in vitro* and *in vivo* as well as for *ex vivo* modification for cell therapy. MC show a superior performance in CAR-T cell therapy research if carrying a *Sleeping Beauty* transposon element, which led to a 4.4-fold increase in the transposition rate when compared to plasmid vectors due to the reduced size of the backbone ("SCAR"). Furthermore, the use of MC in AAV manufacturing could demonstrate a significantly improved quality of the resulting AAV by significantly reducing contaminations of AAV capsids carrying undesired plasmid backbone sequences from about 26.1% to below background levels. Recent trends and progress in pre-clinical studies suggest that the time has come for preparation of such minimalistic vectors in a *High Quality Grade* to enable for example the production of viral vectors for gene therapy. Furthermore, significant developments in transfection efficiency of non-viral vectors suggest that also GMP grade MC will be needed in the near future for direct clinical applications. We like to give here an overview on this circular supercoiled monomeric expression cassette and discuss the advantages and drawbacks of different approaches to produce MC DNA, present recent developments, their applications, and future prospects.

748. Yeast Surface Display Techniques Enhance Development of Chimeric Antigen Receptors

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Chimeric antigen receptor (CAR) engineered T cells have energized the field of cancer immunotherapy with their proven ability to treat CD19+ malignancies in the clinic and emerging efficacy in treating other diseases. The CAR receptor imparts T cells with the ability to recognize antigens independent of MHC. The CAR antigen recognition domain often requires much engineering to generate binding interactions resulting in target-specific activation. Unfortunately, developing a functional CAR with current standard methods is empirical and inefficient, requiring individual cloning of combinations of binding domains (often scFvs) and spacer regions followed by low throughput cell-based and mouse experiments to verify activity. The scFvs incorporated into CARs often derive from published antibody sequences reformatted into scFvs, a process which frequently results in a loss of affinity and/or stability. Additionally, these antibodies are usually of murine origin, raising the risk of immune response that may hamper treatment. Applying humanization techniques can alleviate this concern, but the process is arduous, requiring many iterations to find a suitable variant. This work aims to enhance the efficiency of CAR development by incorporating protein engineering and yeast surface display techniques to facilitate scFv humanization and affinity modulation. Yeast surface display is a genotype-phenotype linkage strategy for functional screening of proteins through tethering to the yeast cell wall by covalent linkage. This linkage allows for facile screening of large combinatorial libraries by magnetic selection, fluorescence-activated cell sorting, or cell-based selections. Importantly, yeast have eukaryotic protein processing machinery capable of producing complex proteins, including antibody-like domains, which are often incorporated into CARs. The use of CAR-like linkers in scFv screening will ensure that isolated scFvs are functional in the desired molecular context (e.g. N- or C-terminal linkage, spacer flexibility, etc.), which can be extremely important to their binding properties. To meet these ends, a novel scFv-IgG4 hinge construct was developed in this work. The antigen recognition domain of a CD19-targeted CAR currently in clinical testing (NCT02051257) was humanized using two distinct methods. Yeast surface display techniques were employed to screen expression, foldedness, and affinity of the humanized variants, adding significant efficiency to the CAR development process that generally requires lentiviral transduction of cell lines to verify these characteristics. The resulting CARs showed comparable activity to the equivalent murine versions in degranulation and internal cytokine staining assays. Ongoing experiments will assess in vivo activity of these CARs in lymphoma models and the need for further affinity improvement. In a second project, a CD123-targeted CAR currently in clinical testing (NCT02159495) was humanized. The resulting clone suffered a 550-fold affinity decrease relative to the murine parent. Error-prone PCR was applied to the humanized variant, and the resulting sublibrary was displayed on the surface of yeast and sorted for variants with increased or diminished affinity. Resulting CARs will be screened for selectivity against CD123+ and CD123-dim cells for effective treatment of AML with minimal damage to healthy tissue. Taken together, this work develops a suite of yeast surface display techniques to streamline the engineering of chimeric antigen receptors, facilitating new adoptive immunotherapies for a variety of diseases while simultaneously evolving new CAR constructs and validating them preclinically.

749. TCR Integration into the TRAC Loci Enhances T Cell Function

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Adoptive therapies using T cells engineered to express a recombinant T Cell Receptor (TCR) have shown promising results in the treatment of some malignancies. Current clinical protocols utilize autologous T cells that are collected by apheresis and engineered with retroviral vectors to stably express the recombinant TCR. Although advances in vector design and codon optimization have improved the expression level of the TCR, this technology faces two major limitations. First, co-expression of the endogenous TCR alpha and beta chains results in TCR mispairing with the recombinant chains which affects the recombinant TCR expression and increases the risk of non-specific TCR. Second, current approaches utilize randomly integrating vectors, including gamma-retroviral, lentiviral and transposons, which all result in semi-random integration and variable expression of the CAR owing to transgene variegation. Position effects may result in heterogeneous T cell function, transgene silencing and, potentially, insertional oncogenesis. Using genome editing, specific disruption of TCR alpha or beta chain followed by the transduction of the recombinant TCR

using randomly integrating vectors prevents the mispairing but not the variegated TCR expression. Here, using CRISPR/Cas9 we present a novel strategy that targets the NYESO-TCR transgene into the constant chain of the TCR alpha or beta. In one single step, we disrupted the expression of an endogenous TCR chain and use its promoter to control the recombinant TCR expression. Using AAV6 to deliver the donor template, we optimized the editing protocol and reached more than 50% TRAC-NYESO T cells. Such approach results in a high, greatly homogenous and predictable TCR expression level, which is similar to the natural TCR. *In vitro,* TRAC-NYESO T cells exhibited higher tumor lysis activity than retrovirally transduced CAR T cells, which augurs favorably for their *in vivo* anti-tumor activity (in progress). Finally, to ensure the complete disruption of the endogenous TCR, we also performed dual targeting in both alpha and beta chains which not only results in higher expression homogeneity but also offers off-the-shelf opportunities. The process we describe here, which combines the uniformity and safety of targeted TCR gene integration, should be useful for the further advancement of TCR-based adoptive T-cell therapy.

Cancer - Oncolytic Viruses II

750. Oncolytic Adenovirus Expressing Decorin, Rad.Dcn, Inhibits Lung Metastasis of Breast Cancer in Immune-Competent Mice

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Breast cancer is the second leading cause of cancer related deaths among the women in United States in 2017. During the advanced stages of breast cancer, a majority of the patients will develop distant metastasis, such as lung and bone metastasis. However, the effective therapeutic strategies for distant metastasis are still lacking. Decorin is a natural inhibitor of transforming growth factor β (TGF-β), which could promote tumor progression via multiple mechanisms at advanced stages. We have previously developed an oncolytic adenovirus expressing decorin, rAd.DCN, in which the oncolytic activity is controlled by telomerase reverse transcriptase promoter (TERTp). In this study, we evaluated anti-tumor responses of rAd.DCN and also explored the possible anti-tumor mechanisms in immune-competent mouse breast cancer (4T1) models. Both intravenous delivery of rAd.DCN and intratumoral delivery of rAd.DCN could inhibit primary tumor growth and prevent lung metastasis in subcutaneous and orthotopic xenograft models. However, in orthotopic xenograft model, intravenous delivery of rAd.DCN lead to more oncolytic viruses retaining in lungs and produced higher level of decorin expression both in tumors and lungs. Thus, a more impressive inhibition of lung metastasis and primary tumor growth was observed by intravenous delivery of rAd.DCN. . Also, oncolytic adenovirus mediated decorin expression - decreased expression of Met and CTNNB1, as well as inhibited epithelial mesenchymal transition (EMT) in tumor tissues of both models via two different delivery rountes of rAd.DCN. In addition, rAd.DCN increased expression of Th1 cytokines, such as interleukin(IL)-2, IL-12 and tumor necrosis factor α (TNF-α), and reduced expression of Th2 cytokines, such as TGF-β and IL-6, in lung tissues. Furthermore, rAd.DCN treatments evoked significant anti-tumor immune cell responses, including up-regulating $CD8^+$ T lymphocytes, inducing expansion and maturation of dendritic cells (DCs) and promoting infiltration of T lymphocytes in tumor tissues. In conclusion, rAd.DCN inhibited tumor growth and lung metastasis via multiple mechanisms, such as inhibiting Met and wnt/β-catenin signaling, and modulating systemic and local inflammation and anti-tumor immune responses. Therefore, rAd.DCN could be developed to a potential approach for treating lung metastasis of breast cancer. YY and WX made equal contribution. LW, HW and PS are corresponding Authors.

751. HSV Oncolytic Vectors (oHSV) Armed with the NKGD2 Ligand ULBP3 Enhances Treatment of Gliomas

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Glioblastoma Multiforme (GBM) is an aggressive brain cancer for which there is no effective treatment. Effective oncolytic vector treatment requires a robust replication of the vector and the ability to trigger an immune response against cancer cells. However the anti-tumor immune responses in GBM are impaired by the development of a tumor micro-environment that discourages active immunity and tumor cells acquire the ability to down-regulate the activation of immune cells and induce an anti-inflammatory state affecting natural killer cells (NK), macrophages and T cells. We discovered that the expression of one of the NKG2DL is exquisitely down-regulated in gliomas, suggesting that tumor cells escape NK killing not only by expressing inhibitory factors but also by blocking the expression of ULBP3. Thus we tested the hypothesis that the expression of ULBP3 from an oHSV could improve the efficacy of treating GBM. In the first set of experiments in vitro we infected primary glioma lines with an HSV-based defective vector and confirmed that ULBP3 expression could specifically activate human NK cells in vitro. We then developed an oncolytic vector armed with ULBP3 and evaluated its efficacy in a xenogeneic murine model of primary human GBM. We observed that this new vector not only had an improved therapeutic profile compare to the parental un-armed vector (80% vs 40% long term responders) but that its efficacy relied on the presence of NK cells. Our preliminary data suggest that it is possible to develop an oncolytic vector that retained the ability to replicate efficiently in vivo while activating an NK immune response.

752. Pharmacological Inhibition of Jak/Stat Signaling Enhances the Therapeutic Index of an Oncolytic VSV Encoding Interferon Beta

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VSV-IFNβ-NIS is a replication competent oncolytic rhadovirus that is in Phase I clinical testing in cancer patients after intratumoral or intravenous administration. The IFNβ transgene restricts viral amplification in normal cells, and activates the immune system to prime antitumor activity, while the NIS gene enables serial imaging of sites of viral replication. The MPC-11 tumor model provides a unique system to study the yin and yang effects of robust viral oncolysis as these tumors are exquisitely sensitive to VSV-IFNβ-NIS; one dose of virus induces rapid tumor lysis, high viremia and high sustained systemic levels of IFNβ, which results in elevated transaminases and thrombocytopenia. Importantly, toxicity is reversible if mice survive beyond 5 days, or if tumor burden is low. Using immunocompetent Balb/c mice bearing MPC-11 tumors, we demonstrate here that combination therapy of VSV with ruxolitinib, a Jak/Stat inhibitor, is not toxic, can protect them from tumor lysis syndrome and high systemic levels of virally encoded IFNβ, resulting in prolonged survival of tumor bearing mice. We determined that elevations in transaminases are correlated with high systemic levels of IFNβ. Using a blocking antibody against type I IFN-receptor, we showed that addition of the antibody 2 days after VSV therapy completely ameliorates toxicity, with complete tumor control. Since there is no clinically approved anti-IFNR antibody, we tested if ruxolitinib, a Jak/Stat inhibitor could be used safely to this model to protect mice from systemic IFN toxicity. Ruxolitinib, given for 10 days at the start of VSV treatment, was highly effective at protecting mice from toxicity. Mice given ruxolitinib and VSV therapy had significantly prolonged survival compared to saline, VSV, or ruxoltinib alone. These preclinical data support combination therapy of ruxolitinib with VSV in the Phase I trials.

753. Novel Approach for Systemic Cancer Therapy with Oncolytic Vaccinia Virus through Evading the Host Immune Response

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Vaccinia virus, once widely used for smallpox vaccine, has been utilized for therapeutic agent of an oncolytic virotherapy. In the therapy, the anti-virus immunity would be a strong inhibitor, but vaccinia virus has a unique infectious form to evade host immunity. The form, named extracellular enveloped virus (EEV), is constructed from normal mature virion covered with host-derived outer membrane. This membrane presents host-derived antigens (such as CD46, CD55, CD59, MHC class I), then EEV spreads via blood stream while evading host immune response. EEV morphogenesis is regulated by viral enveloped protein B5R, and the deletion of its four short consensus repeat (SCR) domain resulted in the increase EEV production. Moreover, SCR domain is known for the target region of host immunity, therefore SCR-deleted EEV would enhance their ability of immune evasion. In the study, higher immune evasive-oncolytic vaccinia virus (VV) were engineered by removing SCR, furthermore their EEV productivity from cancer cell lines and their immune resistance against anti-virus immunity were evaluated in order to utilize as an oncolytic agent. SCR-deleted VV (ΔSCR) showed smaller plaque size than B5R-intact VV (B5R), but there were no any difference in progeny virus production from various kinds of tumor cell lines such as breast, colon, epithelial, hepatocellular, lung, neuroblast, ovary and pancreas. Interestingly, ΔSCR showed a tendency to increase EEV production in ovarian cancer cell lines, and their oncolytic effects of EEV were enhanced than B5R due to improvement of the productivity. ΔSCR-derived EEV also showed higher immune resistance than B5R-derived EEV against the exposure to vaccinia-immunized animal serum. B5R-derived EEV were neutralized by immunized serum, resulting in loss of their oncolytic effects. On the other hand, ΔSCR-derived EEV escaped neutralization and sustained their oncolytic effect under the serum influence. Furthermore, their oncolytic effect and immune evasion were examined by intraperitoneal injection of EEV in tumor-bearing mouse model with or without pretreatment of vaccinia-immunized animal serum. ΔSCR-EEV prolonged the survival regardless of the serum pretreatment. In contrast, B5R-EEV was strongly inhibited by the serum while it increased the survival of mice without the pretreatment. Our data indicated that SCR-deleted vaccinia virus showed higher immune resistance against the EEV neutralizing immunity, and also demonstrated stronger therapeutic effects especially in ovarian cancer cell lines. ΔSCR would have potential for treatment of tumors, even in the immunized patients.

754. Oncolytic Herpes Simplex Virus Type 1 (G47Δ) Enhances the Therapeutic Efficacy of an Anti-EGFR Targeted Therapy in Murine Tumor Model

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Growth signal through cell membrane kinase receptors like epidermal growth factor receptor (EGFR) plays a pivotal role in the progression and invasion of certain types of solid tumors such as head and neck, lung, ovarian, colorectal, bladder and esophageal cancers. Anti-tumor monoclonal antibodies (mAbs) have widely been used for the treatment of various malignancies. Recently, accumulating evidence has shown that not only cell intrinsic factors but also the innate and adaptive immunties play an essential role in exhibiting the efficacy of mAbs. Antigen uptake through opsonization of destroyed tumor cells is associated with enhanced antigen presentation by dendritic cells (DCs). Tumor cell death induced by mAbs will facilitate the uptake of tumor

antigens by DCs, and lead to activation and expansion of tumor-specific T cells. G47Δ, an oncolytic HSV-1 with triple genetic modifications, not only selectively and robustly replicates in and efficiently destroys tumor cells, but also induces systemic antitumor immunity. Cellular proteins and tumor-associated antigens released from destroyed tumor cells are thought to activate DCs and elicit adaptive antitumor immunity. Here, we examined whether G47Δ enhances the therapeutic efficacy of an anti-EGFR antibody using an immunocompetent mouse tumor model. N2a-EGFR, which stably overexpresses EGFR, was made by transfecting cDNA of full-length human EGFR gene to Neuro2a (neuroblastoma) cell line derived from HSV-1 susceptible A/J mouse. A/J mice harboring subcutaneous N2a-EGFR tumors were treated with intratumoral injections with G47Δ, intraperitoneal administration of anti-EGFR monoclonal antibody (cetuximab), or both, and the tumor size was evaluated. The combination therapy of G47Δ and cetuximab significantly inhibited the growth of subcutaneous tumors compared with each therapy alone ($p<0.05$). In a bilateral subcutaneous N2a-EGFR tumor model, intratumoral injection of G47Δ to the left side tumors could reduce the size of uninjected right side tumors more efficiently when cetuximab was used in combination. The enhanced antitumor effect of G47Δ in combination with cetuximab was not evident when tested in athymic mouse, suggesting that T cell immunity was involved in the antitumor effect. In order to further evaluate the mechanism of the combination therapy, tumor infiltrating lymphocytes (TILs) were accessed by flow cytometric analysis. A significant increase in numbers of TILs, especially CD8+ T cells (CD3+ CD8+), and CD8/Treg (CD4+ Foxp3+) ratio was observed in mice that received the combination therapy, when compared with those treated with G47Δ alone. Similarly, the number of DCs also increased in the drainage lymph nodes. Depletion of CD8+ cells eliminated the enhanced therapeutic effect caused by cetuximab when combined with G47Δ, suggesting the involvement of adaptive immune responses. Mice whose subcutaneous N2a-EGFR tumors were cured by the combination therapy successfully rejected a rechallenge by subcutaneous N2a-EGFR implantation. These results demonstrate that oncolytic herpes virus G47Δ enhances the therapeutic effect of an anti-EGFR monoclonal antibody by facilitating the antitumor adaptive immune responses. The combination of G47Δ with antitumor monoclonal antibodies may be a useful strategy for other types of cancer as well.

755. Investigating the Bioenergetic and Metabolic Effects of Oncolytic Measles Virus in a Stromal Model of Cellular Transformation

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Vaccine strain measles virus (MV) is oncolytic in numerous models of malignancy. The mechanism behind the selectivity of MV for transformed cells is poorly understood. To investigate, an established step-wise model of cellular transformation was used, in which progressive oncogenic hits were stably and additively expressed in human bone marrow derived mesenchymal stromal cells (Funes et al., 2007). The most highly transformed cells (5H) were more permissive to productive oncolytic MV infection and cell killing than the less transformed counterparts. Numerous studies have reported an impact of viral infection on host cell metabolism. Therefore, we examined the metabolic and bioenergetic responses to MV infection in transformed and non-transformed MSCs.The Seahorse XF analyser was used to measure oxidative phosphorylation (oxphos) and glycolysis, the two major energy pathways in cells, before and after MV infection. Basal dependence on oxphos and glycolysis increased with transformation. Moreover, spare respiratory capacity (SRC), a measure of energy that can be produced by a cell under stress, indicative of healthy mitochondrial function, reduced with cellular transformation. MV infection had a profound effect on MV-resistant hTERT cells, where basal oxphos significantly increased 24 hours post infection (24hpi) ($p=0.03$). Although MV infection did not have a direct effect on basal oxphos in more transformed cells, SRC increased significantly in 4+V ($p=0.03$) and 5H ($p=0.05$).To further characterize changes in oxphos upon MV infection, mitochondrial biogenesis was assessed using the fluorescent dye TMRM, which diffuses into the mitochondrial matrix. MV infection significantly increased the number of mitochondria per cell at 24hpi in 4+V (16%) and 5H (29%) cells compared to uninfected counterparts. Mitochondrial area increased upon MV infection, but without a clear relationship to the state of transformation.In order to integrate the Seahorse and mitochondrial data, a global metabolomics analysis (CE-MS approach, Human Metabolome Technologies) was performed. At baseline, hTERT and 5H cell lines have distinct metabolic profiles, where metabolites involved in glycolysis and TCA cycle are elevated in hTERT cells relative to 5H cells. MV infection resulted in increased energy production and an overall increase in the metabolic pool in hTERTs, which may account for the increased mitochondrial biogenesis and oxphos seen. Conversely, MV infection of 5H cells did not significantly affect the metabolic pool. There was a significant increase in levels of the antioxidant glutathione in hTERTs in response to MV infection, which remained unchanged in 5H cells, suggesting that glutathione levels may be correlated with susceptibility to MV infection. We assessed whether exogenously added antioxidant, N-acetyl-cysteine (NAC), would render 5H cells resistant to MV infection. Cells were treated with increasing concentrations of NAC, infected with MV and then assessed for cell viability 48hpi. NAC at 10mM reduced MV-specific cell death from 90% to 67%.Altogether, our data suggests that oncolytic MV infection influences the bioenergetics of infected cells in ways that may have therapeutic relevance. Work is ongoing to understand the complex relationship of bioenergetics to cellular transformation and MV-permissiveness.

756. Oncolytic Virotherapy and Cobalt Ferrite Nanoparticles as Breast Cancer Therapy

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Breast cancer remains an incurable disease in the majority of cases, combination therapy seems the best option to target the cancer cells by many different mechanisms. this need smart therapeutic strategies such as oncolytic virotherapy using viruses that replicate selectively in tumor cells and Nanoparticles as novel agents that can target tumor cells

with promising results. Our work is to produce combinational therapy include the use of Newcastle disease virus Iraqi strain and cobalt ferrite NPs to treat breast cancer in vitro and in vivo. **Methods**: cobalt ferrite NPs prepared by co- precipitation to produce stable colloidal nanoparticles. NDV propagated on specific pathogen free embryonated egg and virus quantified using Hemagglutination test (HA) and Tissue Culture Infection Dose 50 (TCID50) method. Nanoparticles were characterized using different methods, Energy Dispersive X-ray spectroscopy (E-DX), X-Ray Diffraction (X-RD), Fourier Transform Infrared (FTIR), Atomic force microscopy (AFM), Field Emission Scanning Electron Microscopy (FESEM), UV-Vis spectroscopy, zeta nanosizer, flame atomic absorption spectrophotometer (FAA). The efficiency of (NDV, and nanoparticles) treatment alone or/and the combination treatment were evaluated as anticancer therapy in vitro using different breast cancer cell lines (AMJ113, MCF-7 and AMN3, SKOV-3) as well as normal cells by MTT cytotoxicity assay and studied this combination using Chou-Talalay analysis for synergism determination. Furthermore, detection of apoptosis pathway by measuring caspase-8 and caspase-9 using fluorescent assay. The cells tested infected with certain multiplicity of infection (MOI) (0.3, 0.1, 0.03, 0.01, 0.003 and 0.001) viral particles. The cobalt ferrite NPs concentrations were (2, 1, 0.5, 0.25, 0.125 and 0.062) μg/ml. The in vivo study used mammary gland carcinoma (AM3)-implanted in female mice as tumor model. Thirty Female mice were transplanted with AM3 tumor cell line, after tumor grew; tumor bearing mice were divided into 6 treatment groups include (NDV treatment only, by cobalt ferrite NPs treatment and combination group and control untreated group. **Results**: The MTT cytotoxicity experiment showed presence of synergism effect between the virulent Iraqi NDV strain and cobalt ferrite NPs on all cancer cell lines tested in most concentrations. the combination of ND virotherapy with Co-Fe NPs was antagonistic and not synergistic at all concentrations when tested on normal cells, therefor normal cells exhibited the highest resistance. Moreover, the results revealed that cobalt ferrite NPs combined with NDV treatment had the most powerful effect on inducing apoptosis in breast cancer cells. The in vivo experiment results showed that all untreated tumors grew progressively without remission. The significant inhibited growth with less total tumor volume in therapeutics groups were in combination group. **In Conclusion**, current study showed that the combination of NDV and Cobalt Ferrite NPs have a synergistic effect on breast cancer in vitro and in vivo and enhance apoptosis induction by both pathways with no toxicity on normal cells.

757. CD20 Targeting of Oncolytic Measles Virus in the Treatment of Adult Acute Lymphoblastic Leukemia

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The Edmonston-B, vaccine strain of measles virus (MVNSe) has oncolytic activity in numerous preclinical tumour models with safety and some clinical efficacy also being demonstrated in clinical trials. Adult acute lymphoblastic leukemia (ALL) is an aggressive malignancy with a poor outcome in 50% of patients. Recently, the GRAALL-2005/R clinical trial showed that anti-CD20 monoclonal antibody, rituximab, improved outcome in CD20-expressing ALL. To determine whether targeting MV entry through the CD20 antigen can recapitulate some of these benefits, we used a MV expressing a scFv against CD20 as a C-terminal extension of the H glycoprotein. Our experiments also used CD20-targeted MV 'blinded' to the native MV receptors. Since neutrophils are key effectors of rituximab and are important in MV oncolysis, we focused our experiments to clarify their role. We generated NALM6 cells expressing 'high' and 'low' levels of CD20. MV replication was measured by $TCID_{50}$ at 24 hr time-points and MV-N mRNA/GADPH by RQ-PCR showing similar growth curves and MV-N mRNA levels for the targeted and parent virus. Infection of NALM6 cells by MVHCD20 virus showed similar levels of killing to MVNSe, and there was significantly greater levels of killing by MVHCD20 of the CD20 positive cells compared to MVNSe (p=0.03). Since it is also possible for the MVHCD20 to enter cells via native MV receptors CD46 and CD150 (SLAM) we investigated the effect of 'blinding' the virus to these. As expected, there was a small, non-significant reduction in efficacy in the CD46 blinded CD20 virus, MVHCD20CD46blind, but no difference with the CD150 blinded virus, MVHCD20SLAMblind, as CD150 is not expressed on NALM6 cells. We have previously demonstrated that neutrophils are important effectors in mediating MV oncolysis. To investigate specific mechanisms, we carried out a FACS-based neutrophil phagocytosis assay in which target cells were labelled with PKH67 and the % phagocytosis determined by the frequency of dual expressing CD15+/PKH67+ cells relative to the total CD15+ cells. This demonstrated that in the presence of anti-MV antibody a similar level of phagocytosis was seen 24 hpi with MVNSe, at a MOI of 1.0, to uninfected CD20 expressing NALM6 cells in the presence of rituximab (53% c/w 58%). The percentage phagocytosis strongly correlated with the anti-MV IgG concentration (Pearson's correlation, r=0.937; p <0.001) The level of phagocytosis was significantly increased when complement was active (student t-test, p=0.008). Recent work on CLL and rituximab showed that this assay does not necessarily indicate true phagocytosis, as only part of the target is internalised by effector cells - a phenomenon known as 'trogocytosis'. To clarify, we prepared an assay as described above and but acquired data with ImageStream$^{®X}$ Mark II. This clearly demonstrated internalisation of small parts of the target cell membrane by neutrophils. This was further confirmed using time-lapse confocal microscopy (Zeiss LSM 700) and with z-stack analysis providing 3-D evidence of internalisation. At no point was phagocytosis seen. In conclusion, CD20 targeted MV has increased oncolytic efficacy *in-vitro* compared to the native MV when infecting CD20 expressing NALM6 cells. Our results show that antibody dependent neutrophil 'trogocytosis' is a possible mechanism of MV oncolysis, a mechanism which has not previously been described in this context. We are currently quantifying its impact. Overall our data suggest that targeting MV entry through CD20 could have efficacy in the treatment of ALL.

758. Engineering Simian Foamy Virus for Safe and Effective Tumoricidal Therapy

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Toca 511 is a replication-competent retrovirus, Murine Leukemia Virus (MuLV), used for Oncolytic Virotherapy (OV). MuLV replicates only in dividing cells, facilitating excellent tumor-targeted infection. Clinical trials with Toca 511 have shown very promising results, however, MuLV has a leukemogenic property in mice, and thus the use of MuLV for OV is associated with potential tumorigenic risks. Spumaviruses, also known as foamy viruses (FVs), are distantly related retroviruses which infect only dividing cells and induce formation of syncytia - large multinucleated cells. In contrast to its widespread prevalence in various mammalian species, FV infection has not been associated with any diseases in their natural hosts. Those biological properties make FVs an attractive safe retroviral platform for OV. The natural preference of FVs to infect dividing cells limits the possible off-target infections, whereas the strong Cytopathic Effect (CPE) caused by the virus can lead to cancer cell death. Indeed, a previous study used Human Foamy Virus, also known as Prototype Foamy Virus (PFV), and demonstrated efficient killing of glioblastoma xenografts in mice. Simian Foamy Viruses (SFVs) infect many different non-human primate (NHP) species and occasional SFV zoonotic infections in humans hunting or butchering NHPs have been reported. However, similarly to the natural hosts, no indications of any disease have been found in the infected humans, suggesting that SFV could be safe to use in humans. We have performed an initial *in silico* characterization of the *env* genes (Env is the primary factor responsible for FV-induced CPE) of PFV, two SFV strains from chimpanzees (SFVcpz) - PAN1 and PAN2 - and other simian and non-simian FVs. The analysis proved that PAN1, PAN2 and PFV *env* show very high nucleotide sequence homology. However, on the amino acid sequence level, PAN1 and PAN2 Env proteins are only 48.3% identical, while PAN2 and PFV Env proteins show much higher amino acid sequence identity (84.3%). We then compared the tumoricidal potential of PFV, PAN1 and PAN2. We found that PAN2 virus showed prominent oncolytic property in various human cancer cell lines, when compared with PFV. We also generated infectious molecular clones from these SFVcpz strains. Our data showed that a recombinant chimeric SFVcpz virus between PAN1 and PAN2 (PAN1/2) killed various human cancer cell lines more efficiently than PAN1 and PFV. These observations suggest that SFVcpz viruses, such as PAN2 or PAN1/2 chimera, can be utilized in a safe and effective tumoricidal therapy. In addition, we were able to generate a replication competent SFVcpz which carries a marker gene, GFP. Thus, SFVcpz virus can be engineered to express marker, immunomodulatory or suicide genes, such as thymidine kinase and sodium iodide symporter (NIS) genes, which may further enhance the utility/oncolytic property of SFVcpz. Here we present our initial characterization of the wildtype and recombinant SFVcpz viruses.

759. Novel microRNA Engineered Oncolytic Virotherapy for Clinical Trial

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In the past decade, oncolytic viruses represent a new class of therapeutic agents to cancer treatment depending on their selective replication within infected cells, induction of primary cell death, interaction with antiviral elements of tumor cells and initiation of host's anti-tumor immunity. In the previous studies, we have demonstrated that coxsackievirus B3 (CVB3) was a potent and novel oncolytic agent with direct lysis of human non-small cell lung cancer cells (NSCLC). And two organ-specific (enriched in muscle and pancreas) miRNAs target sequences were constructed into the 3' untranslated region (3'UTR) of the CVB3 genome (CVB3-miR1&217T) resulting in the markedly reduced occurrence of CVB3-induced pancreatitis and myocarditis. However, non-clinical acute toxicity testing of recombinant CVB3 in mice and monkeys showed mild hematological and histopathological abnormal findings in the highest dose group. To improve its safety profile, microRNA target sequence complementary to miR-34a/c (enriched in normal organs) were inserted into the 3' UTR (CVB3-3A/C), 5' UTR (CVB3-5A/C) or both of these regions (CVB3-53A/C) of the CVB3 genome (CVB3-3A/C). All viruses elicited massive viral lysis of tumor cells *in vitro* and *in vivo*. But the novel recombinant viruses showed reduced replications and cytotoxicity in human normal cells expressing high level of cognate microRNAs. Human tumor-bearing nude mice treated with wild type virus showed remarkable weight loss, but recombinant viruses did not. There were no genetic mutations observed in both of microRNA target sequences in 5' UTR in vitro after 10 serial passages, which indicated that the microRNA target sequence inserted in 5' UTR is more stable than in 3' UTR. As the CVB3-53A/C was more inhibited by microRNA than CVB3-3A/C or CVB3-5A/C, CVB3-53A/C was considered to be better candidate virus for clinical translation. Collectively, tissue-specific microRNA could be incorporated into 3' UTR or/and 5' UTR of CVB3 genome. Replication of the recombinant viruses was demonstrated in tumor but not in normal organs of infected tumor-bearing mice. Our current results suggested CVB3-53A/C would be a promising candidate for safer and effective oncolytic virus therapy.

760. The Efficacy of Oncolytic HSV-1 (G47Δ) for Biliary Tract Cancer in Mouse Models

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The prognosis of biliary tract cancer remains poor. Most patients are diagnosed at advanced stages at which curative surgical resections are not indicated. The efficacies of chemotherapies are also limited, so a

novel therapy is urgently waited. G47Δ is a third generation oncolytic herpes simplex virus type 1 that has triple mutations in the γ34.5, ICP6 and α47 genes of the viral genome. G47Δ infects most types of cancer cells, and the tumor selective replication leads to destruction of host cancer cells. An intratumoral injection is usually the most efficient route of administration for G47Δ, however, it may not be optimal for biliary tract cancer, because it tends to spread along the biliary tree. In this study, we investigate the usefulness of G47Δ for treating biliary tract cancer, and its optimal route of administration. Five human biliary tract cancer cell lines (HuCCT1, OZ, NOZ, TGBC2TKB and TYGBK1) were used to evaluate the cytopathic effects, replication capabilities, and infectivity of G47Δ *in vitro*. Four cell lines except for TYGBK1 were susceptible to G47Δ. In three cell lines (HuCCT1, OZ and NOZ), G47Δ showed good to moderate replication capabilities and infectivity. Subcutaneous tumors were generated in athymic mice using HuCCT1 or NOZ cells, and were treated with intratumoral administration with G47Δ. In both models, treatment with G47Δ significantly suppressed the tumor growth compared with mock. HuCCT1 (cholangiocellular carcinoma) cells were implanted to the liver of athymic mice, and were treated with G47Δ ($5x10^6$ pfu) or mock injected into the liver parenchyma near the spotted tumors under laparotomy 30 days after tumor implantation. The mock-treated mice survived for the average of 138 days (n=5), whereas the G47Δ-treated mice survived for the average of 203 days (n=7), two of which survived more than 400 days. In the peritoneal dissemination model using NOZ (gallbladder cancer cells derived from ascites of a patient), intraperitoneal administration with G47Δ significantly prolonged the survival when compared with mock. The possibility of intrabiliary administration was evaluated *in vitro* by plaque inhibition assay using bovine bile. G47Δ lost activity in bovine bile even when bile was diluted to less than 1 %. These results show that G47Δ exhibits efficacy for biliary tract cancer when administrated intratumorally or intraperitoneally. G47Δ may be a useful therapeutic approach for biliary tract cancer when the route of administration is properly selected.

Cardiovascular and Pulmonary Diseases II

761. Stress-Induced Cardiac Mouse Model of Friedreich's Ataxia Corrected by AAV-Mediated Gene Therapy

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Friedreich's ataxia (FA), an autosomal recessive disorder caused by a deficiency in frataxin (FXN), a mitochondrial protein involved in cellular iron homeostasis, is characterized by neurodegeneration and hypertrophic cardiomyopathy. Cardiac dysfunction is the most common cause of mortality in FA, but in most patients, the cardiac disease is often subclinical and not manifested because the neurologic disease limits muscle oxygen demands. All available mouse models to study the cardiac manifestations of FA involve organs in addition to the heart and/or have a severe cardiac phenotype. To generate a cardiac-specific mouse model of the cardiac manifestations of FA more analogous to the typical FA patient, we created a Cre-Lox recombination cardiac-specific excision of FXN exon 4 to generate a heart-specific FA (HSFA) model that mirrors the mild clinical cardiomyopathy of FA, providing a relevant mouse model to evaluate gene therapy for the cardiomyopathy typically associated with FA. Compared to wild-type littermate controls, HSFA mice had lower levels of FXN mRNA expression in the heart with normal levels of FXN mRNA in skeletal muscle, brain, and liver. The HSFA mice had a 69% decrease in cardiac mitochondrial complex II activity compared to wild-type littermates ($p<0.03$). At rest, the HSFA mice exhibited normal cardiac function as assessed by echocardiogram. In contrast, when the heart was stressed chemically (intraperitoneal dobutamine) littermate control mice exhibit a 26% increase in ejection fraction whereas the HFSA mice increased only 9%, a 62% reduction in stress response ($p<0.0002$). Similarly, there was a 46% increase in the stress-induced fractional shortening in littermate controls but only a 13% increase in HFSA mice, a reduction in stress response of 71% ($p<10^{-5}$). Functional cardiac performance was assessed by inducing physical stress for 20 min using an inclined treadmill followed by an additional 3 min test to observe if the mice kept pace above a mid-line. Littermate controls remained above midline for 148 sec, outperforming the 50 sec attained by the HSFA mice ($p<0.02$). A one-time intravenous administration of 10^{11} genome copies of adeno-associated virus (AAV) rh.10 serotype gene transfer vector expressing human FXN corrected the HSFA stress-induced phenotypes. HSFA mice treated with AAVrh.10FXN exhibited an increase in ejection fraction indistinguishable from littermate controls (34% for treated HSFA mice, 26% for littermate controls, $p>0.05$). Similarly, the stress-induced fractional shortening in treated HSFA mice was 53%, an increase in stress response indistinguishable from littermates (46%, $p>0.07$). In summary, the HSFA mice provide an ideal model to study long-term cardiac complications due to FA and the ability of AAV-mediated gene therapy to correct the stress-induced cardiac phenotype observed in many FA patients.

762. AAV9-Mediated Gene Replacement Therapy of Respiratory Insufficiency in Very-Long Chain Acyl-CoA Dehydrogenase Deficiency

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Very-long chain Acyl-CoA dehydrogenase (VLCAD) deficiency is a disorder of fatty acid oxidation (FAO) that results in severe energy deficiency as well as accumulations of long chain fatty acids in metabolically active tissues. FAO defects present with multi-system involvement, including cardiomyopathy, hypoketotic hypoglycemia, myopathy, and rhabdomyolysis. Patients with $VLCAD^{-/-}$ also have respiratory insufficiency but the exact mechanism of this is unknown. Our aims in this study were two-fold: 1) to characterize

the pathophysiology of respiratory insufficiency in VLCAD$^{-/-}$ mice, and 2) to use AAV9-mediated gene therapy or current standard of care dextrose treatment to improve respiratory function. In order to simulate physiologic stressors that trigger metabolic crises in patients, VLCAD$^{-/-}$ and wildtype (WT) mice were exercised and fasted and then breathing was evaluated using unrestrained whole-body plethysmography (UWBP; EMKA, Scireq). No significant changes in baseline breathing were seen between the two groups. However, following a respiratory challenge with hypercapnia (7% CO_2, 21% O_2, nitrogen balance) VLCAD$^{-/-}$ mice had a significantly lower tidal volume, minute ventilation, peak inspiratory flow and peak expiratory flow (P=0.04). These decreased ventilatory parameters indicate that there is weakness in the muscles of respiration - i.e. the diaphragm and intercostal muscles. Moreover, a decrease in frequency of breaths per minute was observed in both VLCAD$^{-/-}$ mice and WT mice compared to respective mice who did not undergo exercise fasting challenge. Accumulation of intramyocellular lipid droplets in VLCAD$^{-/-}$ animals was noted after the exercise-fasting challenge in the respiratory muscles of the tongue, diaphragm and intercostal muscles by oil-red-o staining. This accumulation was not observed in WT animals. Transmission electron micrographs showed abnormal and disorganized morphology of mitochondria within the diaphragm and intercostal muscles in VLCAD$^{-/-}$ animals. No muscle isotype switching was observed in diaphragm and intercostal muscles. To assess the impact of gene therapy on respiratory insufficiency, VLCAD$^{-/-}$ animals were treated with (*r*AAV9)-VLCAD intravenously via the tail vein prior to the start of exercise fasting challenge and UWBP. To model current clinical guidelines, another group of VLCAD$^{-/-}$ animals received an intraperitoneal injection of dextrose solution immediately prior to UWBP. Both AAV9 treatment and dextrose injection groups, showed increased frequency of breathing comparable to the frequency of animals that did not undergo exercise and fasting challenge, and AAV9 treatment showed improved peak expiratory flow. However, neither treatment improved tidal volume, minute ventilation or peak inspiratory flow. In addition, reduction of lipid accumulation within the respiratory smooth muscles was not observed by oil-red-o staining, but clearance of lipid accumulates was noted in cardiac tissue. Thus, respiratory insufficiency in VLCAD$^{-/-}$ animals appears to be due to both a lack of energy as well as an accumulation of potentially cytotoxic lipid-derivatives in muscles that is exacerbated by fasting and exercise stress. In summary, AAV9 gene therapy corrected phenotypes of VLCAD deficiency brought on by physiologic stress in a mouse model, providing insight into future study endpoints useful for therapeutic development.

763. Widespread Airway Distribution and Phenotypic Correction of Cystic Fibrosis Pigs Following Aerosol Delivery of piggyBac/Adenovirus

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Cystic fibrosis (CF) is a common genetic disease affecting multiple organ systems. Chronic bacterial infections and inflammation in the lung are the leading cause of morbidity and mortality in CF patients. CF is caused by mutations in a gene coding for an anion channel, cystic fibrosis transmembrane conductance regulator (CFTR). Delivery of a functional CFTR gene complements this defect. Our goal is to provide a life-long gene replacement strategy that would be efficacious regardless of the 2000 potential disease-causing mutations. In a vector termed piggyBac/Ad, we combined the efficiency of an adenoviral-based vector with the persistent expression of a DNA transposon-based non-viral vector. Here, we tested piggyBac/Ad in pigs, a large animal model that shares similarities with human lung anatomy. First, we show quantification of widespread viral distribution ranging from 30-50% in the large and small conducting airways of non-CF pigs. Second, we transduced appropriate cell types including ciliated, non-ciliated, basal, and submucosal gland cells. Third, we show phenotypic correction of CF pigs following delivery of piggyBac/Ad expressing CFTR. We measured anion channel activity, airway surface liquid pH, and bacterial killing ability at 5 days post-delivery. In summary, we demonstrate that this vector will: 1) efficiently deliver a transgene to all lobes of a lung in a large animal model, 2) transduce a wide range of respiratory epithelial cell types, including those in submucosal glands, and 3) achieve the gene transfer levels necessary to phenotypically correct a CF pig. In total, we are encouraged that we can achieve efficient CFTR expression from an integrating *piggyBac* transposon system following viral vector delivery.

764. Correction of Half the Cardiomyocytes is Sufficient to Obtain Full Functional Rescue of a Severe Mitochondrial Cardiomyopathy through Cell-Autonomous Mechanisms

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Friedreich Ataxia (FA) is currently an incurable inherited mitochondrial neurodegenerative disease caused by reduced levels of frataxin (FXN). Cardiac failure constitutes the main cause of premature death in FA. While AAV-mediated cardiac gene therapy was shown to fully reverse the cardiac and mitochondrial phenotype in FA mouse models, this was achieved with high dose of vector resulting in the transduction of almost all cardiomyocytes, a dose and biodistribution that is unlikely to be replicated in clinic.

The purpose of this study was to define the minimum cardiac biodistribution thresholds of the therapeutic AAVrh10 vector for the stabilization or full rescue of the cardiac function, in the FA cardiac mouse model. Dose response studies were carried out at two different stages of disease progression. Survival, cardiac function and associated biomarkers were correlated to the dose of vector administrated, the number of vector copies per diploid genome (VCN) and the percentage of cardiomyocyte treated. Full rescue of the cardiac function was achieved when only half of the cardiomyocytes were transduced. In addition, meaningful therapeutic effect was achieved with as little as 30-40% transduction. Furthermore, this therapeutic effect was mediated through cell-autonomous mechanisms for mitochondria homeostasis, although a significant increase in survival of uncorrected neighboring cells was observed. Overall, this study identifies the biodistribution thresholds and the underlying mechanisms conditioning the success of cardiac gene therapy in FA and provides guidelines for the setup of the clinical administration paradigm in large animal. Moreover, these results are particularly encouraging for future clinical translation, since meaningful and full therapeutic rescue of the cardiac function can be achieved with limited transduction coverage of the heart.

765. Current Status of Clinical Development for Gene Therapy with Sendai Virus Vector Expressing the Human Fgf-2 Gene (Dvc1-0101) to Treat Peripheral Arterial Disease

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A new gene product, namly DVC1-0101, has been developed in clinical phase in Japan. DVC1-0101 is a RNA virus vector based on a non-transmissible recombinant Sendai virus vector expressing the human fibroblast growth factor gene (SeV/dF-hFGF2). DVC1-0101 induces 'functional' angiogenesis as well as lymphangiogenesis and thereby expects the effectiveness for peripheral arterial disease (PAD). The first-in-men phase I/IIa clinical trial for 12 patients with severe PAD using the DVC1-0101 was completed. DVC1-0101 was administrated in a four dose-escalation manner up to 5×10^9 CIU/60 kg to one limb per patient. The investigation period was 6 months and follow up period was up to 5 years after the gene transfer. Single dose intramuscular injection of DVC1-0101 demonstrated that this new RNA drug was safe, well-tolerated, and significantly improved walking performance. Subsequently, a phase IIb, randomized, double-blinded clinical trial with intermittent claudication patients began in 2014. The primary objective of this trial is to investigate the safety and clinical efficacy of DVC1-0101 (1 and 5 × 109 ciu/leg) and also aims to examine the dose-response relationship using the rate of improvement in walking as a primary indicator. DVC1-0101 is administrated at 30 sites (15 sites in each upper and lower leg) intramuscularly. The outcomes of this trial will provide valuable proof-of-concept data for DVC1-0101 in PAD patients, and will lead to a better understanding of the cutting edge of gene therapy in cardiovascular application using virus vector.

766. Comparison of Intracoronary versus Peripheral Vein Delivery of AAV for Gene Therapy in a Pig Model

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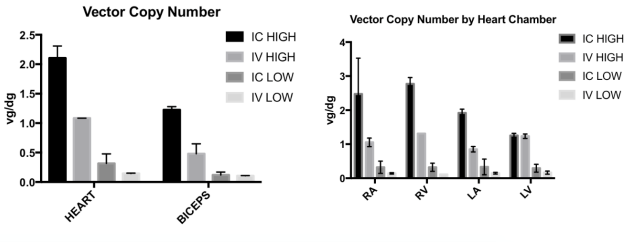
Gene therapy offers a curative option for inherited cardiac mutations that lead to sudden cardiac death in children and young adults, but in many cases will require efficient cardiac delivery of transgenes. Restricting vector diffusion by vessel occlusion can improve viral transduction but presents a risk for triggering arrhythmias and heart muscle damage in an already sensitive patient population. Understanding and optimizing delivery of potential therapies in a large animal model is a key step in translation of pre-clinical results to clinical trials. Using pigs, a standard model for cardiac treatment, our goal was to compare the efficacy of direct infusion into the coronary vasculature (IC) without occlusion and systemic intravenous (IV) infusion. Pairs of ~10 kg minipigs received either 10^{13} (low) or 10^{14} (high) vg/kg of AAV9-CMV-GFP or vehicle by either route. For IC delivery, 70% of the sample was delivered to the left main coronary artery and 30% to the right coronary artery with no vessel occlusion. Animals were euthanized at 18 days post vector administration, when high dose animals began experiencing neurological impairment postulated to result from immune responses to GFP protein expressed in neuronal tissue. Fluorescence microscopy showed generally uniform green fluorescence of low to moderate intensity throughout heart sections from high dose IC and IV treatment animals, with undetectable or very low signal in low dose and control pigs. By the more quantitative and sensitive qPCR detection of vector DNA, both IV and IC treated animals showed dose-dependent and robust gene delivery to the heart (Fig 1A). Notably, IC delivery resulted in a roughly 2-fold higher vector copy number (VCN) in the heart than IV delivery, although, with the high dose, both routes of administration yielded between 1-2 vector genomes per diploid genome in the heart. The VCN from high dose IV delivery (1.08) exceeded that from low dose IC (0.32), indicating the primacy of dose in determining the level of cardiac transduction. Data from the right and left atria and ventricles showed that all 4 chambers received one or more vector copies per diploid genome at the high dose by both delivery routes (Fig 1B). These results provide rationale for systemic delivery of high dose AAV for correction of cardiac diseases.



767. Dual AAV6 Vector Efficiently Delivered Full-Length CFTR Gene and Restored Chanel Function in Primary Human AEC

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Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the cystic fibrosis trans-membrane conductance regulator (CFTR) gene. The resulting lack of functional CFTR protein causes altered balance of ion and water transport and increased viscosity of secretions in a variety of exocrine epithelial cells. In the respiratory airways thick mucus leads to relentless cycle of infection and inflammation with subsequent severe lung damage, which is the main cause of morbidity and mortality in these patients. Recent therapeutic approaches have primarily focused on development of pharmacological correctors and potentiators which are specific only to particular mutations of the gene. Several attempts were made in the past to replace the defective CFTR gene regardless of the mutation type using recombinant AAV as the vector carrying the normal CFTR gene to airway epithelial cells (AEC). Such approach was unsuccessful, in part, because of the large size of the CFTR gene which exceeds the limit of AAV expression cassette. To overcome this limitation, we attempted a recently described novel approach of splitting CFTR gene into two single-strand expression cassettes: (i) carrying CBA promoter and CFTR 1-741aa sequence and (ii) CFTR 742-1481aa sequence followed by polyadenylation site. Alkaline Phosphatase gene was used as the recombination site in each sequence to produce the full-length CFTR gene when both vectors infect the same cell. Each of the 2 sequences was packaged separately in AAV6-WT capsid which we have previously shown to efficiently infect primary human AEC in both submerged and air-liquid interface (ALI) culture. The effectiveness of this AAV6-dual-CFTR in replacing the full and functional gene was tested in two independent experiments. AAV6-GFP was used during both experiments to visualize overall transduction efficiency by AAV6 vectors. **Experiment 1.** Primary human bronchial epithelial (HBE) cells from two CF donors (homozygote delta F508 and a heterozygote delta F508 -R347P) were infected from the apical side with the AAV6-dual-CFTR with total MOI of 50,000 for 12h in Bronchial Epithelial Cell Growth Medium (BEGM). The next day, BEGM was replaced with ALI media keeping the cells submerged until confluence. HBE cells were allowed to differentiate at the air-liquid interface for 4 to 6 weeks before measuring chloride conductance in Ussing chambers and measuring CFTR mRNA expression by qPCR. **Experiment 2**. Similarly, primary human nasal homozygote delta F508 (HNE) cell were infected with AAV6-dual-CFTR with an MOI of 50,000 prior to differentiation in ALI culture. HNE were differentiated for 3 weeks post infection before evaluating channel activity in Ussing chambers. These two experiments demonstrated higher response on treated HBE and HNE cells with AAV6-dual-CFTR vectors when compared to control, and that response was enhanced when inducible short-circuit currents with forskolin were applied. These results support the potential usefulness of such dual vector approach in CF gene therapy. In our future studies we aim to optimize dose, conditions and use of capsid-optimized AAV6 vectors to maximize efficiency of transduction of AEC and ultimately increase expression of functional CFTR. **Acknowledgement:** This project is supported by funding from Katie Rose Cystic Fibrosis Research Fund at the University of Florida.

768. Correction of Respiratory Insufficiency in an ALS Mouse Model Following Intralingual Administration of RrAAVrh10-*miRSOD1*

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Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease with no current cure. Patients with ALS die 3-5 years after diagnosis from respiratory failure. However, if the bulbar muscles and motor neurons are affected first, death occurs within 2-3 years. Bulbar involvement results in dysarthria and dysphagia leading to recurrent aspiration, choking and aggravation of respiratory disease. Approximately 2% of ALS cases are linked to gain of function mutations in the *SOD1* gene encoding the Cu/Zn superoxide dismutase (SOD1). Mutant SOD1 is found aggregated in mitochondria disrupting many cellular processes, specifically in motor neurons. Respiratory insufficiency increases over time in ALS patients as their hypoglossal and phrenic motor neurons begin to deteriorate, and innervation of the tongue and diaphragm is lost. *Our hypothesis is that reducing the expression of mutant SOD1 in the tongue will reduce lingual pathology and in turn improve nutrition, decrease upper airway pathology and impact respiratory function.* To execute this hypothesis, we treated $SOD1^{G93A}$ mice, the most commonly studied ALS mouse model, with an intralingual injection of rAAVrh10 vectors carrying a microRNA against the mutant SOD1. At 8.5 weeks of age, rAAVrh10-*miRSOD1* or PBS was intralingually injected into $SOD1^{G93A}$ mice. Non-transgenic littermate control mice were injected with PBS. Beginning at 13 weeks of age respiration of treated $SOD1^{G93A}$ mice and both controls, was regularly monitored via whole body plethysmography under normoxic conditions and with respiratory challenges of hypoxia (11% O_2) + hypercapnia (7% CO_2). Minute ventilation, a comprehensive analysis of volume inspired per minute, was improved in miRSOD1 treated mice, during the respiratory challenge. Additionally, videofluoroscopic swallow study testing was performed once every two weeks from 3 months of age through end-stage disease. Blinded reviewers analyzed the video for several parameters (i.e. lick and swallow rates). Early results indicate a mitigated dysphagia phenotype in the miRSOD1 treated mice compared to the vehicle treated mice. These functional benefits resulted in increased survival of miRSOD1 treated animals by one week. These physiological improvements are correlated to the presence of rAAV genomes and a reduction in $SOD1^{G93A}$ mRNA. In conclusion, it appears that targeting the tongue has a significant impact on the overall respiratory function of this ALS mouse model and intralingual therapy may be a potential therapeutic target for bulbar insufficiency in ALS patients.

769. Designing Cell Delivery Vehicles with Programmable Degradation

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Introduction: Conventional cell therapy often relies on the single or repeated injection of cells into the body. Stem and progenitor cells, specifically Outgrowth Endothelial Cells (OECs), are poor candidates for cell delivery due to the reduced survival while in solution and the need to have them act at specific anatomic sites during specific time windows. The use of biomaterials as cell delivery vehicles can bypass some of these challenges associated with conventional cell therapy delivery. One focus of research is developing a delivery vehicle that displays on demand biodegradation for the local delivery of OECs for specific time frames at specific doses to enhance new blood vessel formation angiogenesis. Alginate is a naturally occurring biomaterial derived from algae, recognized to be biocompatible while eliciting a low immune response, and most importantly do not interfere with the functional survival of the cells. However, alginate is not enzymatically digested by mammals. An alternative approach to achieving a controlled degradation of the alginate hydrogels is based on the enzymatic degradation by alginate lyase (AL). Nevertheless, it is difficult to exert control over the time frame and extension of alginate degradation in the presence of AL. To overcome this challenge, modified OECs with a lentivector expressing AL upon doxycycline (dox) induction was developed. **Methodology:** HEK293T and OECs (isolated from human umbilical cord blood) were co-transduced with Lv-Dox-AL (lentivector expressing AL upon dox induction) and Lv-eGFP-Luc (lentivector expressing GFP and luciferase). AL expression was performed by qPCR. Two percent low molecular weight (LMW) alginate hydrogels were fabricated both in the form of macroscopic injectable hydrogels, and monodisperse suspensions of microgels. Cells were seeded in 24 wells plate and the alginate disks or microgels were placed above them. The activity of AL was determined by its ability to degrade alginate. Mechanical integrity of the alginate gel was measured using a rheometer. To verify the cells migration from the alginate gel, transduced cells were loaded into RGD-alginate hydrogels or scaffolds surrounded by fibrin gel. At different time points the fibrin and alginate gel or scaffolds were digested and the number of cells was quantified. **Results:** HEK293T and OECs upon dox induction (5ug/mL) showed an expression of AL 10-fold and 100-fold, respectively, more than the no-dox group. With OECs, alginate disks presented almost 60-fold more degradation than the no-dox group. Furthermore, for both HEK293T and OECs, the storage modulus showed a weaker gel for the dox group when compared with the no-dox group. Degradation of the microgels was also observed when in contact with HEK293T expressing AL. HEK293T cells were recorded for 48h, and the images showed that the majority of the microgels shrank in the dox group, while very few of the microgels shrank in the no-dox group.Finally, we tested the capability of HEK293T cells to proliferate and migrate from RGD-alginate scaffolds. The cells were seeded in the scaffolds, and after 3 days the dox group presented double the amount of cells versus the no-dox group. After five days, the dox-group presented more migration from the scaffolds to the media than the no-dox group. The same experiment was performed with hydrogel disks surrounded by fibrin gel. The dox group also presented more proliferation and migration with the alginate hydrogels. **Conclusion:** Alginate hydrogels or scaffolds can be effectively used to deliver cells. This study demonstrated the capability of locally delivering OECs from alginate hydrogels and can be used in a wide variety of applications to enhance current cell delivery methods, specifically in the context of angiogenesis.

770. The Role of Peripheral Blood Derived Outgrowth Endothlial Cells and Their Exosomes in the Progression and Treatment of Pulmonary Arterial Hypertension

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Introduction/Aim: Previously we reported the therapeutic effect of BMPR2 augmented endothelial progenitor cell therapy in a rat PH model. We know there is little direct cell integration into the pulmonary endothelium following intravenous injection, thus we attribute the positive physiological effects of our cell therapy to cell-to-cell communication via secreted factors such as exosomes (exo). In this study we look at comparing exos protein profiles from PAH patient peripheral blood endothelial progenitor derived outgrowth endothelial cells (PB-OECs) and control PB-OECs, and we assess their capacity to interact with and transport BMPR2 into human microvascular endothelial cells (HMVEC). Additionally, we begin to examine functional abnormalities of these PAH PB-OECs compared to control PB-OECs in the presence of hypoxia to further understand the pathological role endothelial progenitor cells play in PAH and we then stimulate the BMPR2 pathway via BMP7 with the aim of reversing these abnormalities. **Methods:** 1. Exosome study- PB-OECs are isolated and cultured from 15mL of PAH or control peripheral blood. Exosomes are isolated via differential centrifugation, and characterised with a NanoSight, TEM and SEM. Exosome protein profile was via mass spectrometry. Exo-Quick™ treated exos and GFP-exos labelled were used to view exo localisation within the target HMVECs both live and fixed via confocal microscopy. For BMPR2 trnasfer studies: cells were either transduced with AdBMPR2 or AdTrackLuc, or untransduced before exo isolations. BMPR2-Exos were incubated on HMVECs for 48hrs before being washed off, and the cells lysed for western blot analysis. 2. PB-OEC hypoxia study- following isolation and culture as state above, PAH and control PB-OECs were incubated in hypoxia (1% O_2) for 5 days. After which, proliferation, apoptosis and eNOS production assays were conducted. PB-OECs were also treated for 48 hours with BMP7 and appropriate protein markers were assessed via western blot and immunofluorescence. **Results:** 1. Exo study- Exosomes were positively identified as 40-200nm via NanoSight, TEM and SEM. Protein profiling of exos showed differential protein expression in the PAH vs control for 326 proteins, including a relative down-regulation of BMPR2 in PAH exos. Exosome interaction with HMVECs demonstrated localisation around the nucleus of the cell,

both in live and fixed samples. Additionally, BMPR2 expression was significantly increased in cells treated with BMPR2-Exos. 2. PB-OEC hypoxia study- under hypoxic conditions, PAH PB-OECs have a significantly higher rate of proliferation compared to control. This is coupled with a higher level of endoglin protein expression. Apoptosis was significantly decreased in PAH PB-OECs compared to control and a decrease was also seen in eNOS production. Additionally, fibronectin organisation is impaired in PAH PB-OECs in both hypoxic and normoxic conditions, however this is somewhat corrected when treated with BMP7. **Conclusion:** PB-OECs and their exosomal secretions may play a major role in influencing the abnormal function of resident ECs in the pulmonary vasculature of patients with PAH. We have previously shown rat derived EPCs augmented to over-express BMPR2 when delivered to the pulmonary endothelium ameliorates disease. This may be due to the restoration of these cells by BMPR2 up-regulation and/ or the delivery of BMPR2 via exosomes to the pulmonary endothelium.

771. An Implication of NOX4 in Pulmonary Vascular Remodeling Contributes to Pulmonary Hypertension in COPD

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Background: Pulmonary hypertension (PH) in chronic obstructive pulmonary disease (COPD) has been suggested as a result of emphysematous destruction of vascular bed and pulmonary hypoxia microenvironment. However mechanisms underpinning the pathogenesis of PH remain elusive. The dysregulated expression of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidases and superoxide generation of pulmonary vasculatures are involved in hypoxia-induced PH. **Methods:** In this study, the involvement of NADPH oxidase subunit 4 (NOX4) in pulmonary arteriolar remodeling of PH in COPD was investigated by ascertaining the morphological alteration of pulmonary arteries and pulmonary blood flow using cardiac magnetic resonance imaging (cMRI), and the expression of NOX4 and its correlation with pulmonary vascular remodeling and pulmonary functions in COPD lungs. **Results:** Results demonstrated that an augmented expression of NOX4 was correlated with the increased volume of pulmonary vascular wall in COPD. Of note, the volume of distal pulmonary arteries was inversely correlated with pulmonary functions, but positively correlated with the main pulmonary artery distensibility, right ventricular myocardial mass end-systolic and right ventricular myocardial mass end-diastolic in COPD patients. In addition, an increased malondialdehyde and a decreased superoxide dismutase was observed in sera of COPD patients. Mechanistically, transforming growth factor-beta (TGF-β) could dynamically induce the expression of NOX4 and production of reactive oxygen species (ROS) in pulmonary artery smooth muscle cells and lead pulmonary arteriolar remodeling in COPD lungs. **Conclusion:** These results thus suggest that the NOX4-derived ROS production may play a key role in the development of PH in COPD by promoting distal pulmonary vascular remodeling.

772. AAV-Mediated Therapeutic Strategy for Ischemic Heart Disease

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Recent work in cellular reprogramming of fibroblasts into cardiomyocytes has demonstrated therapeutic potential for cardiovascular disease, in which viruses were utilized as biomaterials for successful transgene delivery. However, retrovirus- or lentivirus-mediated transgene delivery into the cardiovascular system was majorly used and is not yet feasible for application to humans. Here, we propose to utilize AAV-mediated gene delivery as a cardiovascular regeneration strategy to generate new cardiomyocytes and limit collagen deposition in the injured heart. This was achieved by inducing synergism of Gata4, Mef2c, and Tbx5 (GMT)-mediated heart reprogramming and thymosin β4 (Tβ4)-mediated heart regeneration in cardiac fibroblasts, an *in vitro* myocardial ischemia model. AAV-GMT promoted a gradual increase in expression of cardiac-specific genes, including Actc1, Gja1, Myh6, Ryr2, and cTnT, with a gradual decrease in expression of a fibrosis-specific gene, procollagen type I and here AAV-Tβ4 help to induce GMT expression, providing a AAV-mediated potential next therapeutic cell reprogramming strategy for ischemic diseases.

655. AAV Mediated Gene Therapy Prevents Emphysema in a Novel Mouse Model of Alpha-1 Antitrypsin Deficiency

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Alpha-1 antitrypsin (A1AT) is the major circulating serum antiprotease and normally is secreted by hepatocytes. One prominent function of A1AT is to irreversibly inactivate neutrophil elastase, thus protecting elastin in the pulmonary interstitium from degradation. For the majority of patients with A1AT deficiency, lung disease is the life-limiting manifestation. This study for the first time tests optimized vectors to determine if we can prevent emphysema in a truly deficient in alpha-1 antitrypsin (A1AT-KO) mouse model, which recapitulates the clinical characteristics of human emphysema. Previous work in our lab used the CRISPR/Cas9 system to successfully disrupt all five copies of the serpinA1 gene, leading to mice with undetectable levels of circulating A1AT protein. This mouse model develops spontaneous emphysema, with early signs detectable at 35 weeks of age. We aimed to determine whether rAAV mediated A1AT serum protein augmentation will alleviate the pulmonary phenotype, and is able to prevent the development or progression of emphysema in these mice. Three cohorts of A1AT-KO mice received single treatment

via intravenous delivery of dual-function AAV9.CB-AAT vector at 8 weeks (1st and 2nd cohorts) or at 35 weeks (3d cohort) of age, and evaluation of respiratory function and mechanics outcome measures were assessed at 35 weeks (1st cohort), or 50 weeks (2nd and 3d cohorts) of age. Pulmonary mechanics were measured in age-matched, gender-matched treated and control knockout mice (FlexiVent, EMKA, Scireq). Lung tissue were collected for comparative evaluation of the alveolar diameters. The Pressure-volume loops (PV) of the treated knockouts showed significantly decreased compliance compare to their untreated controls as evidenced by shift of PV curves downwards. Similarly, the quasi-static compliance was significantly decreased in treated mice, which reflects improved intrinsic elastic properties of the respiratory system in treated mice. A single injection with the AAV vector led to life-long expression of detectable levels of normal human A1AT in the serum of A1AT-null mice as determined by ELISA. This indicate that we can safely deliver AAV vectors systemically driving the production of fully-functional normal A1AT protein. These experiments are the the first to show that AAV mediated A1AT protein augmentation is able to prevent the progression of emphysema associated with A1AT deficiency.

Cell Therapies III

774. Repurposing Endogenous Immune Pathways to Improve Chimeric Antigen Receptor T-Cells Potency

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CAR T-cell therapies hold great promise for treating a range of liquid malignancies but are however challenged to access and eradicate solid tumors. To overcome this hurdle, CAR T-cell were engineered to secrete different cytokines known to improve T-cell antitumor activity, prevent T-cell anergy and reduce activation induced cell death. While cytokine-expressing CAR T-cell were shown to be be highly active against solid tumor in *in vivo* models, they have also led to toxicity associated with the systemic release of cytokine. Therefore, new engineering strategies enabling the fine tuning of cytokine secretion by CAR T-cell are warranted. We sought to explore one of these engineering strategies by integrating an IL-12 chimeric heterodimer expression cassette under the control of the endogenous promoters regulating PD1 or CD25. Because both genes are known to be activated upon tumor engagement by CAR T-cells, they could be repurposed to secrete cytokine only in the vicinity of a given tumor. This approach would reduce the potential side effects induced by their systemic secretion while maintaining their capacity to improve antitumor activity. By combining TALEN® technology with AAV6 repair vectors delivering the CAR to the TRAC locus and the IL-12 to the CD25 or PD1 loci, we have engineered CAR and IL-12 expressions under the respective control of TCR and CD25 or PD1 regulatory elements. This double targeted insertion led to the disruption of PD1 and TRAC genes, to the expression of a tool CAR and to the conditional secretion of IL-12 in the media. Such secretion was found to be transient, dependent on tumor engagement and to follow the regulation patterns of CD25 or PD1 genes, commonly observed upon T-cell activation. In addition, it was also found to enhance the antitumor activity and the proliferative capacities of CAR T-cells. Similar results were obtained when IL-15 was substituted for IL-12. This proof of concept paves the way for seamless multi-repurposing of immune pathways to generate smarter CAR T-cells able to sense and react to their environment in a highly regulated and specific manner.

775. Immunomodulation via Ex Vivo MSC Therapy

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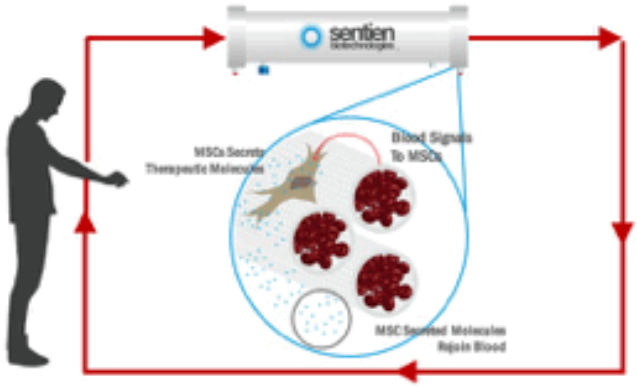
Mesenchymal stromal cells (MSCs) are known to secrete potent molecules and extracellular vesicles contributing to immunomodulation and wound healing. Clinical trials using MSCs as infusion therapeutics have been successful in demonstrating safety, but clear clinical benefit has not consistently been realized. Lack of clinical effectiveness may be due to ineffective dosing of MSCs and short exposure times of the infused cells. Sentien Biotechnologies has pioneered a new way to control the delivery of MSC secreted factors by using an ex vivo cell therapy approach as a novel route of extracorporeal administration. Sentien's lead product consists of a continuous flow bioreactor with MSCs immobilized on the extraluminal side of a hollow fiber membrane. Patient's blood flows through the lumen of the hollow fibers and are conditioned by MSC secreted factors, allowing for the blood cells and MSCs to sense their environment and react to it. In vitro studies were performed to assess MSC function within Sentien's reactors. The technology was also tested as a continuous ex vivo therapy in an ischemia/reperfusion dog model of Acute Kidney Injury (AKI) and an increase in survival was observed in the treated animals. Additionally, toxicological studies performed in healthy dogs verified a pharmacokinetic and pharmacodynamic response to MSCs that was consistent with a potent immunomodulatory mechanism of action. A multi-center, randomized, placebo-controlled double-blind study of extracorporeal MSC therapy has begun in human subjects with AKI receiving continuous renal replacement therapy. Ex vivo MSC therapy using this reactor technology has promise for other clinical applications requiring systemic immunotherapy for tissue repair and regeneration.



776. Efficacy of Cybrocell™ Fibroblast Intradiscal Cellular Therapy for Disc Degenerative Disease

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Introduction: Numerous studies have shown that fibroblasts share various properties with mesenchymal stem cells including: a) regeneration; b) anti-inflammatory activities and; c) antimicrobial activities. CybroCell is an allogeneic preparation of fibroblasts generated under Good Manufacturing Practices (GMP). Our preclinical studies have demonstrated successful treatment of animal models of disc degeneration. **Methods**: 18 subjects with degenerative disc disease where randomized to receive either saline, CybroCell alone, or CybroCell plus autologous platelet rich plasma (PRP). 10,000,000 cells where injected in 1-3 discs in a volume of 1-1.5cc. Safety and efficacy was evaluated based on adverse events, quantified on rating scales and MRI. **Results:** Cell administration was successfully performed with no treatment associated adverse events. Statistically significant improvements in visual analogue score (VAS) and Owstery Disability Index (ODI) where observed for the CybroCell treated compared to Placebo at 3 and 6 months. Specifically, **Baseline** Placebo 7.2 (±1.6), CybroCell 6.2 (± 1.2), CybroCell + PRP 6.3 (±2.8); **3 Months** Placebo 6.6 (±2.0), CybroCell 3.8 (±1.5), CybroCell + PRP 5.4 (±3.9); **6 Months** Placebo 7.0 (± 1.8), CybroCell 3.2 (±1), CybroCell + PRP 4.8 (± 3.3). Similar improvements in the Oswestry Disability Index where observed with CyrbroCell along compared to Placebo. **Baseline** Placebo: 56.7 (±11.6), CybroCell 51.3 (±11.1), CybroCell + PRP 39.5 (±22.2); **3 Months** Placebo 46.8 (±9.7), CybroCell 29.0 (±12.2), CybroCell + PRP 33 (±27.8); 6 Months Placebo 49.8 (±8.4), CybroCell 31.3 (±8.9), CybroCell + PRP 28.5 (±20.5). **Conclusions:** Intradiscal administration of CybroCell in patients with disc degenerative disease is safe and effective at improving pain and disability. Paradoxically, the addition of autologous PRP appears to inhibit efficacy of CybroCel.

777. Genetic Modification of Stem Cells Utilizing Non-Integrative and Autonomously Replicating DNA Vectors

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Introduction: Embryonic Stem Cells (ESCs) are a cellular tool that holds great potential for developing experimental models to understand the mechanisms and to develop treatments for a variety of diseases. Typically, the modification of these cells is done by using integrating viral vectors. Although vectors based on modified viruses are unquestionably the most effective gene delivery systems in use today, their efficacy at gene transfer is, however, tempered by their potential integration and genotoxicity. An ideal vector for the genetic modification of cells should deliver sustainable therapeutic levels of gene expression without compromising the viability of the host in any way. Permanently maintained, episomal and autonomously replicating DNA vectors, which comprise entirely human elements, might provide the most suitable method for achieving these goals. Here we propose a non-viral, non-integrating and autonomously replicating DNA vector system based on the use of a Scaffold Matrix Associated Region (S/MAR). This technology can persistently genetically modify Stem Cells (SCs) cells without causing any molecular or genetic damage. **Results:** Murine embryonic stem cells (mESCs) were stably labeled with GFP-S/MAR DNA vectors, which yielded robust and persistent levels of transgene expression. The S/MAR DNA vectors remained episomal and did not modify the stem cells' properties, as demonstrated by the expression of pluripotency markers such as Alkaline Phosphatase, Nanog, Oct4 and SSEA-1. The behavior of the vector during differentiation was then evaluated *in vitro* in a random differentiation experiment, in which GFP-expressing representatives of the three germ layers were obtained. Finally, the suitability of S/MAR DNA vectors was challenged *in vivo* by generating chimeric mice, which showed robust and sustained transgene expression in the majority of the organs analyzed, independently of their embryonic origin. **Conclusions:** For the first time, we demonstrate that the non-integrating non-viral DNA vectors we have developed, can be used as tool to genetically modify both murine and human SCs. We have shown that the DNA vector system provides robust transgene expression without integration, which is sustained through the differentiation process without causing any damage or affecting the pluripotency of the cells. As an ultimate demonstration of the capability of the DNA vectors, we show that the genetically modified SCs we have produced are capable of contributing to the cells and tissues of transgenic mice and that transgene expression is sustained into chimeric tissues. We hope this technology can be applied as a tool for the episomal gene and cell therapy of genetic diseases.

778. Genetically Engineered Natural Killer Cells for Cancer Immunotherapy

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Over the last decade Chimeric Antigen Receptor (CAR) based T cell therapy (i.e. CAR T) has seen great success for the treatment of cancer. However, CAR T technology has several shortcomings and clinical success has largely been limited to blood cancers. Challenges facing CAR-T therapy include immune evasion through loss of target antigen expression by tumor cells and inhibition of CAR T function by tumor expressed inhibitory molecules such as PD-L1. A more ideal approach would use a cell type that can perform both antigen dependent (i.e. CAR) and antigen independent killing of cancer cells, such as Natural Killer (NK) cells. NK cells have demonstrated antigen specific killing when engineered to express CARs and they also mediate direct killing of transformed cells with reduced or absent MHC expression, i.e. antigen independent killing. Additionally, NK cells can carry

out antibody dependent cell mediated cytotoxicity (ADCC) on cells bound by appropriate antibodies via their CD16 receptor. In light of these multiple modalities for cancer cell killing, there is a surging interest in NK cells for cancer immunotherapy. However, the use of unmanipulated NK cells to treat cancer has shown limited efficacy; except for some rare instances, such as KIR mismatch. For instance, members of our team have performed clinical trials and generated preclinical models of ovarian cancer that are partially responsive to NK therapy. These preclinical models are ideal for testing enhanced NK treatments as they are partially responsive to standard NK therapy but are unable to eradicate tumors, in part due to inherent NK inhibitory receptors and tumor mediated NK cell inhibition. Thus, we hypothesized that NK therapy could be fully realized, and potentially even rival CAR T therapies, through the use of genetic engineering. However, numerous previous reports demonstrated that NK cells have an extreme intransigence to genetic modification. Nonetheless, through a large amount of optimization studies, we have achieved consistent gene knockout frequencies of 80-99% in primary human NK cells using the CRISPR/Cas9 system. We have knocked out several negative regulators of NK cell function, including TIGIT, CISH, ADAM17 and PD1, in primary human peripheral blood NK cells. We further performed functional assays *in vitro* and *in vivo* using human cancer cell lines and demonstrate that our genetic modifications results in enhanced NK function against cancer cells. Moreover, we have also employed homology directed repair and CRISPR/Cas9 base editor technology to modify CD16 to a cleavage resistant form, which leads to enhanced ADCC. In addition to optimizing gene editing, we have developed novel 'NK' CARs that activate NK cells significantly better than T cell CARs by using domains found in NK cell activating receptors. In order to deliver these CARs to NK cells with high frequency, we optimized site-specific transgene delivery using CRISPR/Cas9 and rAAV. Using an EGFP reporter system, we have demonstrated consistent site specific integration in primary NK cells exceeding 75%. Our preliminary data strongly supports the notion that NK based cancer immunotherapy can be fully realized by using genome engineering. Furthermore, since NK cells are not associated with graft versus host disease, as T cells are, they are highly suited for use in allogeneic settings and therefore hold significant clinical potential as an off-the-shelf cellular product.

779. Ex Vivo Characterization and Delivery of Stromal Cell Secreted Signaling to Attenuate T-Cell Activation

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Mesenchymal stromal cells (MSCs) can attenuate pathologic immune response in the absence of cell-cell contact through secreted signaling factors that include extracellular vesicles. This indirect immunomodulation has been well demonstrated in the suppression of T-cell activation. Poor therapeutic efficacy has been observed in vivo when administered during periods of disease remission. It is thus becoming increasingly important to evaluate how MSCs are administered, where they localize, what tissue signaling is present to activate MSCs, and what cell numbers and persistence are expected in a local compartment. A systems framework that accounts for the composite effects of an engrafted stromal cell cluster within a defined microenvironment can begin in the definition of spatiaotemporal factors that can influence an immune response. Using a compartmental microvenvironment systems model, we have notably uncovered several key findings towards improved MSC understanding with practical implications. Our bioassay system involes a mitogen activated T-cell population in transwell co-culture with MSCs which allows us to specifically evaluate the effects of soluble communication and subsequent outcomes. Strategic and controlled system perturbations led to the following major findings: (1) an improved MSC quantification method with predictive cross-study aplicability, (2) a direct association between MSC immunomodulation and system volume, (3) a minimum duration of MSC - T-cell communication for effective MSC immunomodulation, (4) the requirement for heterotypic cross-talk between activated T-cells and MSCs to realize a modulating effect with association to several activating mediators (IFNy, TNFa, IL17) liberated by T-cells. An improved understanding into the governing microenvinroment interactions between MSCs and T-cells allowed us to then exploit and engineer conditions to harness and deliver this potent MSC therapeutic in a highly controlled and scalable system. To this end, we have designed and implemented a dynamic hollow-fiber bioreactor system that facilitates soluble factors and exosomal transfer from MSCs to target immune cells in an ex vivo setting. MSC bioreactors were characterized for MSC viability and secreted function before being tested in a T-cell suppression assay under continuous flow. Under continuous co-culture, we observed a clearl suppression of T-cell activation under bioreactor conditions akin to static transwell conditions. Extracellular vesicles were measured in these studies and found to contain RNA transcripts with trans-signaling potential. Taken together, this pharmacological modeling approach can further drive the field forward through insights into the dynamic interplay between engrafted cells and their local microenvironment. Understanding these interactions and their limitis can define critical specifications for the delivery of MSC therapy and be engineered into new drug delivery formulations for improved therapy.

780. Universal Xenogeneic CAR T Cells: A Novel Translational Platform Using Gene Editing of Human Cart Cells to Target Canine Tumors

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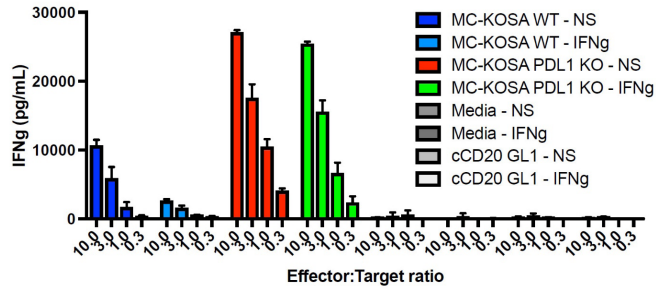
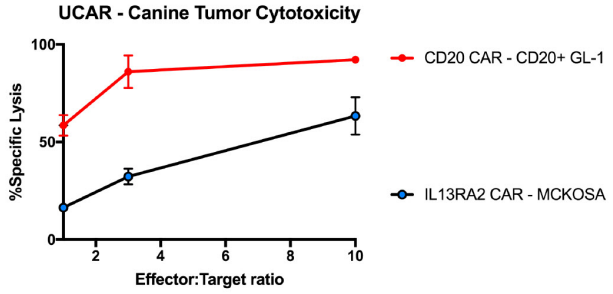
Autologous CAR-T cell have proved to be a potent therapy for hematological malignancies, but custom-made cell products for each patient is a serious obstacle for widespread use. Similarly, heavily pretreated patients with refractory hematological tumors are often leucopenic with anergic T cells. Emerging literature suggests universal CAR-T cells (UCARs) as an alternative manufacturing program to build off-the-shelf CAR T cell banks from healthy donors. To achieve this goal, UCARs must evade host-mediated immunity, avoid graft-versus host reactivity while exerting anti-tumor effects. The first instance of multiplex gene-editing to generate human UCARs, using CRISPR/Cas9 and simultaneous lentiviral CAR transduction,

was tested in immunodeficient mice, but GVHD and HVG reactions may not correlate to an allogenic transplant in humans. We initiated evaluations of UCARs in an immune competent dog model, which is increasingly recognized for its relevance for translational research in humans. We detected no preformed antibodies in canine serum that could trigger hyperacute rejection of transplanted human UCARs. We performed one-way mixed lymphocyte reactions to assess immune rejection by canine T cells and observed a reduction in xenoreactivity by canine T cells cultured with MHC-disrupted human cells. Surprisingly, target-specific human UCARs were significantly better at lysing canine leukemia cell lines than canine osteosarcoma cell lines (Fig. 1), and we hypothesized that this could be due to significant homology of checkpoint receptors between the two species. We investigated physical and functional interactions of human PD1 with canine PDL1 by generating canine PDL1-KO leukemia and osteosarcoma cells, which were confirmed through genomic sequencing and through staining with human PD1-Fc after canine IFN-gamma stimulation. Interestingly, stimulation of target-specific human UCARs was not different between PDL1+ and PDL1- leukemia, but was significantly increased between by the PDL1- osteosarcoma when compared to stimulation by the PDL1+ osteosarcoma cells. The stimulation of UCAR by PDL1+ osteosarcoma cells was further inhibited after pre-stimulation of the tumor cells with canine IFN-gamma, which we confirmed causes an upregulation of PDL1 gene expression (Fig. 2). These data support the utilization of human UCARs in the treatment of canine tumors and suggest that human checkpoint inhibitors and/or gene-editing of checkpoint receptors can be evaluated for safety and efficacy in the canine model. Using this platform, the speed and mechanism of UCARs rejection can also be modeled *in vivo* and strategies to extend the durability and persistence of UCARs can be developed.





781. Decorin Modified Umbilical Cord Mesenchymal Stem Cells (Mscs) Attenuate Radiation Induced Pulmonary Injuries via Regulating Inflammation, Fibrotic Factors and Immune Responses

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The lack of effective therapeutic approaches for radiation induced lung injuries (RILI) has been well recognized. Genetic modification of mesenchymal stem cells (MSCs) has been explored as a mean to enhance their functions and emerged as a potential tool for diverse lung diseases. In this study, we armed MSCs with replication deficiency adenovirus, Ad(E1-).DCN, to produce decorin-expressing MSCs, MSCs.DCN. In an experimental animal model of RIPI, MSCs.DCN and control MSCs modified with Ad(E1-).Null (MSCs.Null) were engrafted via intravenously injection at 6h after radiation. We found that both of MSCs treatments could alleviate histopathological injuries, including reducing lymphocytes infiltration, decreasing apoptosis, increasing proliferation of epithelial cells, and inhibiting fibrosis at the later phase. However, MSCs.DCN produced much more impressive therapeutic effects. Moreover, we analyzed the potential mechanisms of MSCs.DCN mediated therapeutic responses. We discovered that MSCs treatments reduced the expression of chemokines and inflammatory cytokines, while increased expression of anti-inflammatory cytokines both in peripheral blood and local pulmonary tissues. Importantly, MSCs mediated expression of decorin still could be detected in pulmonary tissues at 3 month after radiation, suggesting MSCs homed to injured lungs after administration. Although both MSCs.DCN and MSCs.Null could down-regulated target genes of decorin, such as transforming growth factor (TGF-β) and vascular endothelial growth factor A (VEGFA), MSCs.DCN was much more effective in inducing IFN-γ expression and inhibiting Col3α1 expression in pulmonary tissues, as well as decreasing proportion of regulatory T cells (Tregs) in peripheral blood. Furthermore, we treated radiated mice with MSCs.DCN and MSCs.Null at 28 days after exposure, and our data suggested that treatment at acute phase after radiation evoked much stronger responses both in attenuating inflammation and in inhibiting later fibrosis. In conclusion, DCN modified MSCs could attenuate acute inflammation after radiation and inhibit later fibrosis significantly. Likewise, DCN enhanced the functions of MSCs via targeting pro-fibrotic factors and Tregs. Therefore, MSCs.DCN holds the promise as an effective cell therapy approach for treating RILI. DL and FK contributed equally to this paper. QZ, YY and HW are corresponding authors.

782. Capture and Release of High Purity Functional T Cells via Phase-Change Hydrogel Microspheres

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Introduction Cell purification unit operations heavily influence cell therapy process efficiency and outcomes. Most positive selection processes utilize magnetic beads, which are difficult to rapidly remove and prohibit multiple surface marker-based sorting. We have developed a magnetic bead-free Hydrogel Phase Change-Enabled Release for Cell Separation platform (HyPer-Sep) which captures specific cell populations from heterogeneous samples, rapidly releases high purity captured cells, and enables multiple marker-based cell separation. ***Methods*** An ionotropic hydrogel was formulated and used to form hydrogel microspheres, then conjugated to streptavidin. Release buffer with EDTA was shown to rapidly dissolve microspheres. Mesh-based devices were tested for optimal retention of microspheres and bound cells and elution of contents following application of release buffer. Biotinylated antibodies against CD34, CD3, CD4 and CD8 were used with microspheres. Multiple microsphere sizes were evaluated for efficiency of cell capture and release. Flow cytometry was used to assess biotin binding, cell count, viability and phenotype. For activation studies, CD4+ and CD8+ cells isolated from PBMCs were activated with CD3/CD28 hydrogel microspheres. Cells were expanded in X-Vivo 15 medium for 9 days. Flow cytometry was used to assess cell activation and expression of phenotypic markers. ***Results*** Streptavidin-conjugated microspheres ranging in size from 20 μm to 200 μm diameter were fabricated. First, Kg1A cells were incubated with biotinylated anti-CD34 and streptavidin-conjugated hydrogel microspheres (30 μm diameter), then loaded onto a mesh filter (20 μm pore). 85% of the Kg1A population were captured and released using hydrogel microspheres. We applied this system to capture and release CD4+ or CD8+ T cells from a CD3+ cell population, with purities of 97% and 99% respectively, and viability >99%. Performing this experiment using PBMCs resulted in purities of 96% (CD4+) and 97% (CD8+). Finally, we performed sequential separations of CD4+ and CD8+ cells from PBMCs. Unbound cells from the first separation (CD4-) pass through the mesh filter device while bound cells (CD4+) are retained. Unbound cells were applied to a second mesh-based device for CD8+ cell separation. Upon release, highly purified populations of CD4+ (97% purity) cells and CD8+ (98% purity) cells were recovered (>99% viability). After 48 hours of exposure to activation reagent, CD4+ cells and CD8+ cells showed an increase in activation markers (CD25+ >60% and CD69+ >50%). Cell growth was monitored over 9 days and both CD4+ and CD8+ cells underwent >6 population doublings. ***Conclusions*** HyPer-Sep efficiently captures target cells from heterogeneous samples, rapidly releases high purity/viability bound cells, and enables multi-surface marker-based purification. HyPER-Sep thus provides an alternative cell separation technology to magnetic or immunofluorescent labelling. These releasable hydrogel microspheres are scalable, automation-compatible, compliant with USP<1043> cell therapy manufacturing guidance, and amenable to cGMP manufacturing. This technology offers the potential to eliminate magnetic beads from cell separation unit operations.

783. Defining the Optimal FVIII Transgene for Placental Cell-Based Gene Therapy to Treat Hemophilia A

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The delivery of FVIII through gene- and/or cellular platforms has emerged as a promising approach for HA treatment. Herein, we investigated the suitability of Placental Cells (PLC) as cellular delivery vehicles for FVIII. Human PLC were isolated based on surface c-Kit expression, and expanded *in vitro*. Flow cytometry showed PLC were positive for CDs 29, 44, 73, 90, and 105, and negative for CDs 14, 31, 34, and 45, and HLA-DR. Further characterization by flow cytometry and confocal microscopy established that PLC (passage 3-15) expressed vWF. PLC also constitutively produced and secreted readily detectable levels of endogenous FVIII (0.3±0.2 IU/10^6 cells/24h). Since these constitutive levels of FVIII are not predicted to provide sufficient therapeutic benefit in the context of severe HA, we next assessed PLC's suitability as a transgenic FVIII production platform. We compared the transduction efficiency, FVIII production, and FVIII secretion from PLC following transduction at an identical MOI (7.5) with lentiviral vectors (LV) encoding 4 different FVIII transgenes: 1) a bioengineered human-porcine hybrid FVIII (ET3); 2) a liver codon-optimized ET3 (lcoET3); 3) a liver codon-optimized human FVIII (lcoHSQ), and a myeloid codon-optimized ET3 (mcoET3). Following transduction at equivalent MOIs, PLC were analyzed by flow cytometry and confocal microscopy to measure transduction efficiency and FVIII production, and their conditioned media assayed by aPTT to quantitate FVIII activity. The flow cytometric and confocal analyses revealed that cells were transduced at different levels, despite using an identical MOI. Analysis of the culture supernatants by aPTT also demonstrated marked differences in the secretion of functional FVIII following transduction with each of these vectors. PLC transduced with ET3, mcoET3, lcoET3, and lcoHSQ LV secreted 27.5, 11.8 , 4.06, and 0.52 IU of FVIII/24h/10^6 cells, respectively. Of note is that PLC population doubling time was not affected by transduction with any of the vectors; nor were phenotype or expression of signaling molecules involved in innate immunity, such as TLR 3,4,7, or 8, or in genotoxic stress MICA/MICB ULBP-1. In conclusion, our findings show that PLC possess multiple unique characteristics that make them ideally suited for delivering a FVIII transgene to treat HA. Our studies also highlight the importance of engineering the sequence of the FVIII transgene, not only to optimize mRNA stability/translation but, also to ensure its efficient secretion from the transduced cells into the circulation to provide sufficient therapeutic procoagulant benefit.

784. Development of a Clinical-Grade Meganuclease for Allogeneic CAR T Cell Production

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Chimeric Antigen Receptor T cells (CAR Ts) are revolutionizing the treatment of hematologic malignancies. Autologous (patient-derived) therapies, though effective, are challenging to produce. Allogeneic (donor-derived) CAR T cells may be an attractive alternative if they can be modified to prevent graft-vs-host disease (GvHD). We previously reported an efficient genome editing strategy to produce allogeneic CAR T cells by targeting the insertion of a CAR transgene directly into the native TRAC locus using an engineered meganuclease and an AAV donor template (MacLeod, et. al., 2017). The resulting cells are CAR+ and do not elicit GvHD by virtue of having the native T cell receptor gene knocked-out by the CAR transgene. To better understand the extent and impact of nuclease activity on the CAR T cell product, we developed a high-sensitivity assay for off-target cutting that takes advantage of the 3' overhangs generated by meganucleases. Based on this analysis, we were able to engineer a second-generation nuclease with greatly reduced off-target activity. By comparing the performance of the optimized nuclease to the parent, we found a surprisingly robust relationship between nuclease specificity and CAR T cell performance. These results suggest that monitoring and control of off-target nuclease activity is critical to optimizing the overall fitness and function of gene edited CAR T cells.

785. Successful Treatment of Refractory Cytomegalovirus Colitis after Haploidentical Hematopoietic Stem Cell Transplantation Using CD45RA+ Depleted Donor Lymphocyte Infusion

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Introduction: Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative treatment for high-risk acute leukemia, whereas there is a risk of severe infections and graft-versus-host disease (GVHD). Recently, HSCT with CD45RA+ naïve T cell-depleted stem cell grafts was reported to reduce the incidence of chronic GVHD, while preserving rapid T cell recovery and transfer of protective virus-specific immunity. Moreover, CD45RA+ depleted donor lymphocyte infusion (DLI) showed a promising result of an effective antiviral boost after haploidentical HSCT. Herein, we report on the successful treatment of refractory cytomegalovirus (CMV) colitis after haploidentical HSCT by CD45RA+ depleted DLI. **Case:** A 21-year-old female patient with relapsed acute B lymphoblastic leukemia underwent a haploidentical HSCT using post-transplantation cyclophosphamide from mother. Diarrhea occurred on day 55, and methylprednisolone was administered under the impression of GVHD. Because watery diarrhea did not improved by GVHD treatments, colonoscopic biopsies were performed on day 68, which revealed CMV colitis. Ganciclovir induction and subsequent foscarnet induction treatment could not improve her colitis, and neutropenia occurred by drug-induced bone marrow suppression. We planned CD45RA+ depleted DLI to treat the refractory CMV colitis and augment immune recovery on day 87. Using a closed bag system, whole blood from donor was separated by density centrifugation to obtain a leukocyte enriched cell fraction for further processing with the CliniMACS system. According to the manufacturer´s protocol, CD45RA+ cells were depleted with CliniMACS CD45RA reagent. The $\log_{10}$ CD3+ CD45RA+ cell depletion was -1.57, and $0.5x10^6$ CD3+ cells, $4.5\ x10^4$ CD3+ CD45RA+ cells, $33.5\ x10^4$ CD3+ CD45RO+ cells per body weight (kg) of recipient weight were infused. The patient did not experience any acute complications, acute or chronic GVHD. Diffuse wall thickening involving entire colon was gradually improved, and CMV pp65-specific cytotoxic T lymphocyte was observed on ELISPOT assay after 4 weeks from DLI. The patient was discharged on day 138, and is on disease-free status for 11 months from HSCT. **Conclusion:** We report a case with refractory CMV colitis after haploidentical HSCT, to whom CD45RA+ depleted DLI was safely administered. This approach can be an effective tool for the improvement of antiviral immunity, while not increasing the risk of GVHD after HSCT.

786. Cardioregenerative Effects of Angiomune™ Allogeneic T Regulatory

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Introduction: T regulatory cells are characterized by immunomodulatory properties. AngioMune is a placental T regulatory cell population optimized for stimulation of angiogenesis. Previous studies demonstrated efficacy in treatment of animal models of limb ischemia. Assessment of angiogenic capacity was assessed in a murine model of heart failure. **Methods**: AngioMune was generated using cord blood CD4+ CD25+ expanded on placental mesenchymal stem cells. Cells where administered epicardially in infarct area 24 hours subsequent to left anterior descending artery ligation. Fractional shortening and remodeling was assessed by echocardiogram 4 weeks after infarct. Assessment of fibrosis and angiogenesis was performed by immunohistochemistry. **Results:** Reproducible generation of clinically-relevant AngioMune concentrations was accomplished, which maintained angiogenic activity after expansion. Administration of AngioMune cells post LAD ligation resulted in smaller infarct size compared to control saline. Echocardiographic studies revealed preserved cardiac geometry and contractility in the treated groups. Decreased infarct size and reduced fibrosis were observed in the AngioMune treated group. Reduction in fibrosis and augmentation of neovasculature was observed by immunohistochemistry. **Conclusions:** AngioMune can be generated in scalable quantities and maintain activity in vitro. In vivo studies in a clinically relevant model of heart failure indicate therapeutic activity. AngioMune is a promising candidate for clinical development in the cardiovascular space.

787. Benefits of Hypoxic Culture of Umbilical Cord-Derived Mesenchymal Stem Cells for Use in Cell-Based Therapies

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BACKGROUND Mesenchymal stem cells (MSCs) represent an attractive avenue for cell-based therapies targeting degenerative diseases. MSC *in vitro* expansion is required in order to obtain therapeutic numbers during the manufacturing process, culture conditions are known to impact cellular properties and behavior after *in vivo* transplantation. In this study, we aimed at evaluating the benefit of long-term hypoxic culturing of umbilical cord derived mesenchymal stem cells on cell fitness and whole genome expression and discussed its implication on cellular therapies targeting orthopedic diseases. **METHODS:** Umbilical cord derived mesenchymal stem cells (UC-MSCs) were isolated from fresh umbilical cord tissue and were cultured side by side in atmospheric (20% O_2) and hypoxic (5% O_2) oxygen partial pressure for up to 3 passages. Clonogenic potential, cell surface marker identity and differentiation potential were studied. Whole genome expression was performed by mRNA sequencing. Data from clonogenic potential, flow cytometry and gene expression by quantitative PCR were analyzed. **RESULTS:** Long-term hypoxic culturing of UC-MSCs had positive effects, as evidenced by an increased clonogenic potential and improved differentiation potential towards adipocyte and chondrocyte lineages. No difference in osteoblast differentiation or in cellular identity were observed. Only a small subset of genes (34) were identified by mRNA sequencing to be significantly dysregulated by hypoxia. When clustered by biological function, these genes were associated with chondrogenesis and cartilage metabolism, inflammation and immunomodulation, cellular survival, migration and proliferation, and vasculogenesis and angiogenesis. **CONCLUSIONS:** Long-term hypoxic culturing positively impacted UC-MSCs fitness and transcriptome, potentially improving inherent properties of these cells that are critical for the development of successful cellular therapies. Long-term hypoxic culturing should be considered for the *in vitro* expansion of UC-MSCs during manufacturing of cellular based therapies.

789. Improvement in Cell Growth through Novel Media Formulations

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Cell therapy technologies have experienced exponential growth due to advances in genetic engineering. This has led to a need for corresponding advances in the media formulations that are used to recover, sustain and expand these important cells. Cell therapy researchers and manufacturers alike, spend valuable time and resources optimizing media formulations to enhance cell proliferation while maintaining functional phenotypes of stem cells and immune cells. Serum free (SFM) and/or chemically defined formulations are thought to be superior due to the appearance that they are safer and more consistent. However, proliferation rates and cell durability often suffer due to the lack of physiologically relevant protein sources and concentrations. Therefore, there is a need for new media supplements to help the cell therapy market thrive. Physiologix™ XF Human Growth Factor Concentrate (hGFC) is a cell culture media supplement that can be used in place of standard serum supplements such as fetal bovine serum or human serum with traditional basal media such as DMEM/F12. In this study, Physiologix™ XF was compared to various SFM. For CD4+ T cells, PBMC were isolated from three donors using standard protocol. CD4+ T cells were further isolated using EasySep hCD4+ T cell Isolation kit. Cells were labeled with CFSE, activated with anti-hCD3/28/2 and cultured over 7 days in RPMI, Glutamax and 2% hGFC or SFM. Cells were collected on days 1,2,4 and 7, stained with Ghost Dye 780 for viability gating on flow cytometer and assayed using CFSE to determine proliferation. Cells were also assayed using flow cytometry for activation markers CD25 and CD69 and exhaustion marker PD-1 to determine the affect on growth in different media formulations. Bone marrow derived mesenchymal stem cells (BM-MSC) were purchased from ScienCell Laboratories. Culture conditions included DMEM/F12 supplemented with 2% hGFC or SFM. Cells were assayed using standard Cell Titer Glo. Induced pluripotent stem cells (iPSC) were purchased from Thermo Fisher Scientific. Culture conditions included DMEM/F12 supplemented with 2% hGFC or SFM. Cells were assayed using Cell Titer Glo. Batch consistency was explored using 5 different manufactured lots of Physiologix™ XF supplemented into standard DMEM/F12. BM-MSCs were grown for seven days and assayed using Cell Titer Blue. Physiologix™ XF was also used as a supplement with an off the shelf SFM to see if this amplified the cell growth further than using conventional basal media.

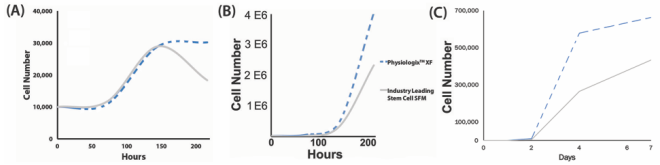

Fig. 1 shows the difference in overall cell number after a week of culture in commercially available basal media supplemented with Physiologix™. Fig. 1A shows the BM-MSC grow in a similar fashion compared to commercially available SFM for the first few days after which the proliferation becomes markedly increased in the Physiologix™ condition. Similarly Fig. 1B-C depicts a similar trend in iPSC and CD4+ T cells. The batch consistency experiments revealed that there was little substantive difference in functional performance across 5 lots. Over 7 days, the BM-MSCs showed very similar growth profiles. The implications of this data are particularly important for those in cell therapy manufacturing where proliferation rate and consistency are critical. Cell growth is not the only characteristic of importance to those in cell therapy manufacturing. Future work is aimed at ensuring that cells grown in Physiologix™ XF media maintain phenotypic fidelity and functional capabilities.

790. High Fold Expansion Platform of Human Pluripotent Stem Cells in a Bioreactor

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Enabling human pluripotent stem cell-base therapies requires innovative solutions to close the gaps that exist between research and

commercialization. These gaps include aspects of cell therapy product quantity, quality, comparability and cost. While translational research aims to make a process that is feasible for early clinical phases, it lacks, in many cases, commercialization viability, especially in terms of quantity and cost. Here we show a highly efficient hPSC microcarriers based expansion platform, with fully animal-free components, in a stirred tank bioreactor. Moving from traditional, 2D, cell culture platform to suspension culture platform, greatly supports large cell quantities while keeping or reducing cost. Likewise, a bioreactor is a scalable expansion system, enabling comparability between different vessel sizes, and therefore between early clinical phases that requires a certain amount of cells and commercialization, which requires significantly larger amount. Bioreactor platforms also enable reduced manpower, reduced deviations, and higher quality, as they are for the most part closed, automated, controlled and monitored. While aggregate based bioreactor platform for hPSC expansion exist, they have many disadvantages. Including the need to perform passaging mid-process and the need to use ROCK inhibitor. The unique attributes and advantages of this microcarrier based platform include a low cell density at seeding, while achieving high fold expansion and obtaining high cell densities at the end of the process without the need for cell passaging. The expanded hPSCs retain characteristics and features of hPSCs cultured in 2D, expressing hPSC-associated markers and genes, stable karyotyping, and differentiation capability. Moreover, we show the feasibly of a seed train from one bioreactor vessel to another, which eliminates the extensive 2D culture needed for seeding large bioreactor volumes. Using this platform during translational research phase, will facilitate the viability of the cell therapy product commercialization, especially for clinical indications that require high hPSC numbers, such as cardiovascular indications.

791. Microcarrier-Based Xeno-Free Expansion of Human Mesenchymal Stromal Cells in a Single-Use Stirred-Tank Bioreactor

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Systems for isolation and expansion of mesenchymal stem/stromal cells (hMSC) from tissues using Xeno-free media are under development and some commercially available platforms are already becoming available from various vendors. The current paradigm for expansion of large numbers of allogeneic cells utilizes planar technologies such as T flasks, cell factories, or hollow fiber devices. These platforms can be useful for generation of small numbers of cells but are of limited utility when large batches of cells are required. Over the past decade, much data has been generated demonstrating the feasibility of using microcarriers in suspension cultures for expansion of hMSC, however, many systems still use media that contains fetal bovine serum. Here we identified microcarriers that support Xeno-free expansion of cells in a single-use stirred tank reactor. Performance of commercially-available microcarriers and a novel Xeno-free microcarrier type were examined in small scale spinners. Microcarriers that promoted cell attachment and growth were selected and conditions that supported growth were optimized. The optimized spinner conditions were implemented in a single-use bioreactor which contains a bottom-mounted impellor. Cell attachment and growth on 6 different microcarrier types were examined. Microcarrier performance of selected microcarrier types was compared to a high performing collagen-coated microcarrier. Results from this study demonstrate that hMSC can be successfully propagated on Xeno-free microcarriers in a single-use stirred tank reactor. Xeno-free protein-coated microcarriers provided the highest cell numbers (**0.46** B cells/L) followed by SoloHill® collagen-coated microcarriers (**0.32** B cells/L) and SoloHill® Star Plus (**0.25** B cells/L). Standard cell characterization assays revealed that cells retained critical quality attributes indicative of cell type. These studies provide the basis for efficient and reproducible generation of high quality cells in a Xeno-free system.

792. Allogeneic CAR T Cells with Multiple Therapeutically Favorable Edits Can Be Created Efficiently Using CRISPR/Cas9

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Remarkable therapeutic benefit of CAR T cells has been observed for hematologic tumors across multiple indications and with different antigen targets. The most advanced systems are lentivirus derived autologous CAR Ts as seen with the approval of Kymriah and Yescarta and the reported clinical trial data from CAR Ts targeting BCMA. Despite these significant advancements, there remains (as ever in oncology) scope for improvement; in this case around supply and consistent product as well as the usual efficacy and safety profiles. Allogeneic (off the shelf) CAR T cells created using gene editing techniques offer the opportunity to improve all of those aspects. Indeed TALEN based gene editing has been used to generate "off the shelf" CAR T therapeutics targeting CD19. However, the CRISPR/Cas9 system provides an unprecedented opportunity to rapidly improve the properties of CAR T therapeutics to treat solid tumors. Using CRISPR/Cas9 gene editing, homology based guide RNAs can be assayed for functionality within weeks so that the most relevant targets can be validated. Furthermore, T cells are very tolerant of multiplex CRISPR based editing, including knock-out and knock-in editing events. Here we show selection of multiple candidate T cell edits that improve T cell function without damaging T cell properties.

793. Towards a New Paradigm for Regulatory and Global Collaboration in Gene and Cell Therapy

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Gene and cell therapies represent the implementation of advanced technologies in clinical settings with the promise of a new paradigm of precision-health and hope for billions across the world. However,

regulatory and ethics approvals for such therapies remains a significant challenge. Typically, such approvals are restricted to a few large markets, and promising therapies are often prohibitively expense for patients. Furthermore, the evidence-base to support many advanced cellular and gene therapies is limited: data from cellular therapies offered in international settings are not widely shared within the medical and research community. We need a new paradigm for collaboration involving all aspects of the production, manufacture, research and clinical application of gene and cellular therapies. In this presentation, we will discuss several paradigms and approaches for the design, development and implementation of a more collaborative, modern, holistic global regulatory framework. We will focus on the need for more inclusive perspectives, broader adoptions, and swifter, more efficient implementation strategies in the interest of furthering an improved educational bedrock and advancing the field. Our hypothesis and analysis suggests that a more integrative and unified regulatory landscape will likely require a multi-disciplinary approach that leverages advances in accelerating technologies and systems-thinking in order to capture and share clinical outcomes across many different geographies and regulatory jurisdictions. It will also require us to draw on diverse experiences and ethical models in order to overcome bureaucracy and make promising gene and cellular therapies more widely accessible and cost-effective.

Gene Targeting & Gene Correction III

794. Delivery of a Hyper-Accurate Cas9 from *Neisseria meningitidis* and its Guide All-In-One Adeno-Associated Virus (AAV) Vector for Efficient In Vivo Genome Editing

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The field of gene therapy has benefited tremendously from the development of CRISPR-Cas9 genome editing. Most applications to date have exploited the Cas9 ortholog from *Streptococcus pyogenes* (SpyCas9, 1,368 amino acids), which has shown considerable promise in *in vitro* and *ex vivo* applications. Yet, its *in vivo* usage has been hampered by its large size, which makes all-in-one (guide RNA + SpyCas9 protein) delivery by potent gene delivery shuttles like adeno-associated virus (AAV) unattainable. Our lab co-developed a different Cas9 ortholog from *Neisseria meningitidis* (NmeCas9), which was the first compact (1081 amino acids) Cas9 to be validated for human genome editing[1-2]. Importantly, NmeCas9 has been found to be naturally hyper-accurate[3-4], with little or no off-targeting detectable even without the extensive re-engineering that was required to improve SpyCas9 accuracy. Finally, the first natural Cas9 "anti-CRISPR" inhibitors were discovered and validated with the NmeCas9 system, enabling off-switch control over NmeCas9 activity[5]. Here, we establish that NmeCas9 genome editing can be accomplished via all-in-one AAV delivery in mammals. Two genes were targeted in the livers of mice: proprotein convertase subtilisin/kexin type 9 (*Pcsk9*) as a phenotype-based target, and *Rosa26* as a negative control. PCSK9 functions as an antagonist to the low-density lipoprotein (LDL) receptor and limits LDL cholesterol uptake, providing a direct functional readout of efficient NmeCas9 editing. The plasmid backbones were packaged in the hepatocyte-specific AAV8 serotype, and a dose of 4×10^{10} genomic copies (gc) per mouse was injected via tail vein. Targeted deep sequencing revealed efficient indel formation at both loci, leading to significantly reduced expression of PCSK9 protein in the liver as well as reductions in serum cholesterol levels. The *Rosa26*-targeted mice did not exhibit these phenotypes. Additionally, potential off-target sites identified in mouse hepatoma Hepa1-6 cells using genome-wide unbiased identifications of DSBs evaluated by sequencing (GUIDE-seq) showed minimal off-target cleavage in mice by targeted deep sequencing. This discovery establishes NmeCas9 as an efficient *in vivo* genome editing tool via all-in-one AAV delivery, thus increasing the repertoire of available Cas9 nucleases for potential therapeutic applications. **References:** 1. Hou et al., 2013. 2. Esvelt et al., 2013. 3. Lee et al., 2016. 4. Amrani et al., 2017. 5. Pawluk et al. 2016.

795. Human MicroRNAs Enhancing Homologous Recombination for CRISPR-Cas9 Mediated, Cardiac Gene Editing

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The CRISPR-Cas9 technology holds the promise to allow precise and efficient manipulation of mammalian genomes, thus permitting correction of monogenic disorders. Reaching this ambitious goal, however would require not only precise introduction of a DNA cut, as CRISPR-Cas9 does, but also damage correction through homologous recombination (HR) using and exogenously administered DNA template, all at a therapeutically relevant efficiency. Unfortunately, the HR machinery is largely less efficient than error prone non-homologous end joining (NHEJ) in mammalian cells, in particular in adult post-mitotic tissues, such as heart and brain. With the purpose of enhancing HR after CRISPR-Cas9 cleavage, we designed an in vitro fluorescence-based assay for the genome-wide, high-throughput identification of RNA regulators and enhancers of precise gene editing. By screening a library of 2,042 human microRNAs mimics (miRbase 21) we identified 11 molecules that markedly increased HR events. Of notice, 10 of these miRNAs belong to two conserved miRNA families sharing the same seed sequence. Analysis of the mechanism of action of these miRNAs revealed that a distinctive feature of their effect is the increase in the intracellular levels of key proteins of the HR machinery. In collaboration with L. Carrier, Hamburg, Germany, we aim at correcting an autosomal dominant genetic defect in a mouse model of a human hypertrophic cardiomyopathy (HCM), caused by a point mutation in the sarcomeric MYBPC3 protein. AAV vectors, engineered to deliver all gene-editing components (Cas9, sgRNAs, HR template), were able to efficiently transduce KI-mouse neonatal cardiomyocytes in vitro and to correct the disease-causing point mutation at a significant level. Importantly, these gene-editing vectors were able to produce genomic rearrangements at their target site in the heart when injected systemically in mice at early neonatal stages. Further enhancement of

the extent of gene editing could be achieved thorough the combined delivery of the pro-recombinogenic miRNAs. Together, these results indicate that precise gene correction can be achieved through the manipulation of the endogenous HR machinery of cardiomyocytes following the delivery of CRISPR-Cas9 components.

796. ITR-Seq: a Novel Method for Detecting Off-Target Activity of Genome Editing Nucleases *In Vivo*

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The adeno-associated viral (AAV) vector genome can integrate into host DNA after treatment with radiation or restriction enzymes, suggesting that AAV might integrate into sites of DNA breakage. In genome editing experiments involving AAV-mediated expression of nucleases such as SaCas9 and meganucleases, we discovered that corresponding recombinant AAV vector sequences integrated into the host genome in proximity to the nuclease target-region where the enzymatic activity of the nuclease might create a DNA strand break. Since AAV integration may also occur in nuclease off-target regions, we hypothesize that the number and location of AAV insertions in the cell genome can be used as an indication of nuclease-specificity. We therefore developed a sensitive, genome-wide method to identify the locations of AAV integration in the setting of gene replacement therapy or genome editing. This method is called inverted terminal repeat (ITR)-Seq, since it uses the AAV-ITR as a starting point to sequence the contiguous host DNA. Experiments were performed to identify the spectrum of off-target indels using this approach. Briefly, total DNA is extracted from tissues of animals administered AAV in the setting of SaCas9/gRNA or gene-specific meganuclease expression. After shearing and adapter ligation, DNA is used as a template for PCR amplification using adapter-specific and AAV-ITR-specific primers. Using next-generation sequencing and bioinformatics analysis, we sequenced the resulting amplicons and identified multiple sites where AAV-ITR integration events occurred. ITR-Seq allowed us to identify AAV integration in cells treated with recombinant AAV that expresses or does not express different nucleases. Analysis of the ITR-Seq results revealed that the on-target region of the corresponding nuclease was one of the most abundant AAV integration sites. Furthermore, most of the off-target sites identified by GUIDE-Seq *in vitro* were also identified with ITR-Seq *in vivo*, while a larger number of off-targets were identified by ITR-Seq only. Insertion and Deletion (Indel) frequency in ITR-identified locations was higher in tissues treated with nuclease-expressing AAV than in control tissues, supporting the notion that ITR-Seq predicted sites where the nuclease induced a DNA break. ITR-Seq has the potential to identify nuclease on- and off-target and AAV integration events in virtually any animal model or human sample. This method may be useful for investigating the safety of AAV-mediated genome editing and gene therapies.

797. Generide™, a Novel AAV Strategy to Treat Pediatric Patients with Methylmalonic Acidemia

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Canonical recombinant adeno-associated virus-based vectors (rAAV) show therapeutic promise in disease models and in clinic. However, current rAAV technology is not feasible to treat pediatric patients, due to episomal genome dilution in growing tissues that leads to progressive loss of therapeutic efficacy. We have developed a novel technology, GeneRide™ that allows to expand the utility of rAAV vectors to treat childhood diseases that require early intervention. GeneRide™ is a promoterless, nuclease-free genome editing technology (Brazel et al, Nature, 2015). Combined with highly liver-tropic AAV vectors, GeneRide™ harnesses the natural process of homologous recombination to integrate the therapeutic gene site-specifically into the *Albumin* (*Alb*) locus in a non-disruptive manner. Following GeneRide™ treatment, expression of the therapeutic gene is linked to that of Albumin via a 2A peptide. Methylmalonic acidemia (MMA) is a rare pediatric genetic disorder typically caused by mutations in a mitochondrial localized metabolic enzyme, methylmalonyl-CoA mutase (MUT). MMA effects appear in early infancy and often result in irreversible neurological outcomes. Treatment options for MMA patients are very limited and liver transplantation is becoming an attractive approach. We recently used a GeneRide™ vector to rescue neonatal murine models of MMA (Chandler et al, Molecular Therapy, 2017). Here, we translate this observation from the rodent model to human cells. In order to establish HR-mediated integration at the human *ALB* locus, we constructed a human specific GeneRide™ vector containing the human MUT coding and homology arm sequences. After application of the vector on both human cell lines and primary hepatocytes, we observed site-specific integration and the consequent MUT expression. This study provides the first evidence that GeneRide™ can mediate efficient genome editing of a therapeutic transgene into the *ALB* locus in human primary hepatocytes and paves the path of developing novel therapeutics for MMA patients as well as for other inborn errors of metabolism.

798. Highly-Sensitive Quantification of Genome Editing Events Using Next Generation Amplicon Sequencing

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Quantifying genome editing events can be technically challenging, especially when editing occurs at low frequency. Here we describe a simplified system which can accurately measure edited alleles at frequencies below 0.5%. Next generation amplicon sequencing services are readily available,

inexpensive, and amenable to high throughput sample analysis. We theorized this service could be adapted for quantifying CRISPR/Cas9-generated indels. To test this method, PCR primers were designed to amplify a 440 bp region surrounding a CRISPR target sequence in the mouse Cypor gene. Primers were designed with unique barcodes to differentiate amplicons from up to 50 test samples. Amplicons from 15 mouse genomic DNA samples were pooled and submitted for Illumina paired-end 150 bp Hi-Seq analysis, generating 7 million reads. Tags with indels were distinguished from wild-type tags by the presence or absence of a 6-nucleotide wild-type Cypor sequence surrounding the CRISPR PAM sequence. This method, termed Indel Detection by Amplicon Sequencing (IDAS), was evaluated using mouse genomic DNA samples with known indel frequencies as determined using alternate quantification methods. The samples were from three groups of mice treated with a CRISPR plasmid by hydrodynamic tail vein injection. The first group ("controls," n=3) received a CRISPR plasmid lacking a guide RNA. The second group ("non-selected," n=3) received a CRISPR plasmid targeting the Cypor gene. A third group ("selected," n=5) received the Cypor CRISPR plasmid followed by treatment with hepatotoxic levels of acetaminophen which causes expansion of Cypor-deficient hepatocytes in vivo. Liver genomic DNA from the three mouse groups was analyzed for Cypor indel frequencies using IDAS and an alternate quantification method, tracking of indels by decomposition (TIDE). In addition, Cypor targeting efficiency was estimated using direct detection and quantitation of Cypor-deficient hepatocytes using immunoflourescence (IF). The IDAS method showed significantly higher indel frequencies in the non-selected vs. control mice (2.03% vs 0.55%, p=0.01). These data were consistent with IF data, which showed 1% and 0% Cypor-deficient hepatocytes in the two groups respectively. In contrast, TIDE analysis showed no significant difference in measured indel rates between the non-selected and control mice, as both groups were within background indel detection levels (2.2% and 2.5%). All three methods showed similar rates of Cypor gene targeting in the selected group, with values ranging from 38 to 45%. These data show the IDAS method accurately measures indel rates with significantly higher sensitivity than TIDE analysis. The method is straightforward, economical, and amenable to high throughput analysis, making it well-suited for quantifying low-efficiency gene targeting events.

799. In Utero CRISPR/Cas9 Deletional Gene Editing in the Mtmg Mouse Model

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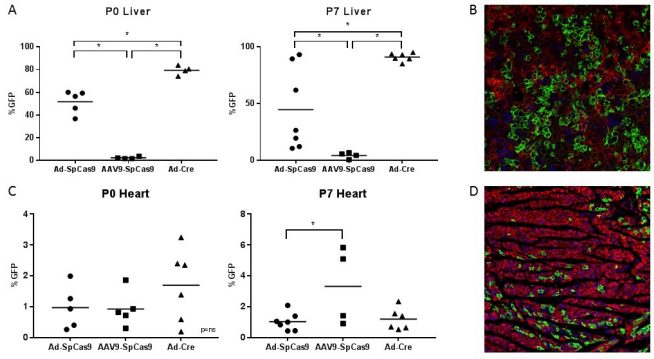
Purpose: The advent of CRISPR/Cas9 genome editing presents an unprecedented opportunity for therapeutic gene correction. In utero application offers the potential to take advantage of the accessibility and proliferative nature of fetal progenitor cells, as well as correct congenital monogenic disorders before disease onset. The heart and liver are attractive targets as congenital genetic disorders of these organs can be diagnosed before birth and often result in significant morbidity/mortality shortly. The mTmG mouse model constitutively expresses a red fluorescent transgene that converts to green fluorescent protein (GFP) expression after Cre recombinase exposure. In this study, we evaluate the feasibility of prenatal CRISPR/Cas9 deletional gene editing in the mTmG mouse model and compare the efficiency of editing following intravenous (IV) delivery of CRISPR/Cas9 via adenoviral (Ad) and adeno-associated viral serotype 9 (AAV9) vector. **Methods:** Streptococcus pyogenes Cas9 (SpCas9) and gRNAs targeting the loxP sequences surrounding the mT gene were delivered in an Ad or AAV9 vector via the vitelline vein into gestational day (E) 16 heterozygous mTmG fetuses (Ad n=11, AAV9 n=9). Due to the limited capacity of AAV9, a split SpCas9 with intein mediated joining was delivered via two AAV9 vectors. E16 mTmG fetuses injected IV with Ad-Cre served as positive controls (n=10). Pups were harvested on postnatal day (P) 0 and 7. Hepatocytes and cardiomyocytes were assessed for editing by PCR, flow cytometry, and confocal microscopy. Statistical analysis was performed with one way ANOVA. **Results:** PCR at all timepoints demonstrated the expected band indicative of successful deletion of the mT segment in the heart and liver using all three vectors. At P0, flow cytometry demonstrated significantly more hepatocyte editing following Ad-SpCas9 compared to AAV9-SpCas9 injection (Figure). In contrast, there was a low efficiency of editing in cardiomyocytes at P0 following both Ad and AAV9 mediated delivery of SpCas9. The improved hepatocyte editing following Ad-SpCas9 vs. AAV9-SpCas9 at P0 persisted through P7. Surprisingly, there was significantly more cardiomyocyte editing at P7 for AAV9-SpCas9 as compared to Ad-SpCas9. Fetuses injected with Ad-Cre demonstrated increased hepatocyte editing when compared to Ad-SpCas9 and AAV9-SpCas9 at both P0 and P7, while cardiomyocyte editing was similar. Confocal microscopy confirmed GFP expression on individual cell membranes following Ad-SpCas9 and AAV9-SpCas9 injection consistent with the flow cytometry results. **Conclusions:** As proof-of-concept, we have demonstrated successful gene editing following in utero delivery of CRISPR/Cas9 constructs in the mTmG mouse model. Ad vector mediated delivery of SpCas9 was significantly more efficient at hepatocyte gene editing, but AAV9 demonstrated improved cardiomyocyte editing after 1 week of life. These findings lay the groundwork for future studies of prenatal CRISPR/Cas9 treatment of congenital monogenic disorders.



A) Cytometry of P0 and P7 liver injected intravenously on E16.5. B) Representative confocal microscopy of a P7 liver edited with Ad-SpCas9. C) Cytometry of P0 and P7 liver injected intravenously on E16.5. D) Representative confocal microscopy of a P7 heart edited with Ad-SpCas9.
* p<0.05

800. rAAV6 Outperform ssODN as Homology-Directed Repair Donor Template for Gene Editing at the B-Globin (HBB) Locus

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Gene editing outcomes following designer nuclease cleavage are strongly impacted by the mode of donor template delivery. When editing at an exon, the rates of homology-directed repair to non-homologous end joining (HDR:NHEJ) can decisively alter clinical outcomes. Sickle cell disease (SCD) is caused by a single nucleotide transversion in exon 1 of the *HBB* gene, resulting in a glutamic acid to valine substitution at the 6th amino acid (E6V). This change increases the hydrophobicity of the adult globin (β^A) and renders it susceptible to polymerization resulting in the characteristic sickling pattern of erythrocytes. Sickle patients remain transfusion-dependent with increased morbidity and a reduced life-span. Gene editing with a nuclease in the presence of a donor template (either recombinant adeno-associated virus (rAAV) or single-stranded donor oligonucleotides (ssODN)) can fix mutations and drive template-driven repair by the cellular repair machinery. For optimal benefit, clinical gene editing in SCD would lead to efficient donor-directed nucleotide change while concurrently limiting on target HBB nuclease-driven gene disruption via NHEJ. To evaluate the impact and clinical relevance of rAAV6 and ssODN delivery in correcting sickle cell disease, we introduced the E6V sickle mutation into human mobilized peripheral blood CD34+ cells (hPBSCs) using Crispr/Cas9 ribonucleoprotein (RNP). Our proof-of-concept study focuses on comparing two donor delivery strategies; rAAV6 with 2.2 kb homology arms (HA) and ssODN comprising of 168 nucleotides. We evaluated the efficiency of HDR in comparison to residual NHEJ rates following Crispr/Cas9 RNP generated double-stranded breaks. Upon *in vitro* testing, the rates of HDR:NHEJ on day 10 post-editing was 31%:18% across 5 CD34+ donors tested using rAAV6 and 13%:30% across 3 CD34+ donors tested using ssODN (~ratio of 2:1 vs. 1:2, respectively). The edited CD34+ cells were differentiated for 2 weeks into erythroid cells and the amount of sickle globin (β^S) in the erythroid precursors were quantified using RP-HPLC. The amount of β^S was 18-27% (n=5) with rAAV6 template delivery and 0-9% (n=3) using ssODN delivery, respectively. Thus, rAAV6 appears to be a superior method for introducing a targeted nucleotide change within the *HBB* locus under these conditions. The *in vivo* relevance of these alternative modes of template delivery is currently being evaluated in the NSG-W41 mouse model that allows for the development of the erythroid compartment in the bone marrow. Engrafted human CD45+ cells within the bone marrow will be assessed for both the HDR:NHEJ ratio and for induction of β^S as a functional outcome of alternative donor delivery methods. Overall, our findings highlight the importance of measuring both HDR:NHEJ ratios and therapeutic protein levels as critical metrics in assessing the potential for clinical gene editing designed to introduce a nucleotide change while limiting on-target gene disruption.

801. Designer Epigenome Modifiers Enable Robust and Sustained Gene Silencing in Clinically Relevant Human Cells

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The human immunodeficiency virus (HIV) is a major global health burden which has claimed over 25 million lives in the past 30 years. The CCR5 co-receptor is necessary for HIV entry into host cells and individuals that are homozygous for inactivating mutations in the *CCR5* gene are largely protected from infection. Therefore, this receptor has gained interest as a possible target for gene therapy against HIV infection. We hypothesize that transcriptional repression via epigenetic modification of the *CCR5* promoter may provide a safer alternative to inactivate *CCR5* expression as compared to genome editing since the genomic sequence remains unchanged and the severity of off-target effects may be reduced. We have generated a panel of transcription activator-like effector (TALE)-based repressors (rTALEs) and TALE-based designer epigenome modifiers (T-DEMs) and tested their activity in a reporter cell line harboring an integrated *EGFP* expression cassette under control of the *CCR5* proximal promoter. While both platforms were able to efficiently modulate reporter gene expression, transient expression of *CCR5*-specific T-DEMs resulted in fast and efficient *EGFP* silencing that remained stable long term. Analysis of CpG methylation via bisulfite sequencing revealed that the T-DEM-induced methylation spread about 2 kb from the target site and was maintained for at least one month, highlighting the potential of T-DEMs in inducing long-term epigenetic modifications which are maintained during cell division. *EGFP* expression could be restored after treatment with the demethylation agent 5-AZA-2'-deoxycytidine or by overexpression of a VP64-based transcriptional activator demonstrating that the targeted epigenetic marks are reversible. To highlight the translational potential of T-DEMs, their functionality was tested in $CD4^+$ primary T cells on two different endogenous targets, namely CCR5 and CXCR4. We observed a significant reduction in gene expression levels at both loci as determined by quantitative RT-PCR up to twenty-one days after the delivery of T-DEM-encoding mRNAs and a corresponding decrease in cell surface protein levels assessed via flow cytometry. Next-generation bisulfite sequencing revealed up to about 25% increase of CpG methylation at both target site compared to controls. Importantly, no changes in methylation profile could be detected at the top 10 predicted off-target sites and RNA-seq analysis revealed undetectable changes in global gene expression. This study establishes T-DEMs as an efficient and safe epigenome editing tool and hence as a novel strategy towards a safe therapy to treat HIV infection.

802. Optimizing AAV6 Transduction of Lymphocytes to Increase Targeted Genome Editing

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Adoptive cell therapies have become a prevailing means to introduce modified cells into patients to treat a number of diseases. In particular, harnessing B cells' ability to produce large quantities of antibodies as an *ex vivo* protein replacement platform is a promising approach for expression of therapeutic transgenes using novel site-specific gene addition techniques. While targeted nucleases can be efficiently introduced into primary cells as mRNA or protein/RNA complexes to achieve high levels of double-stranded DNA break formation, the delivery of a partner DNA 'donor' template required to direct homology-directed repair (HDR) can be a limiting step. We and others have previously shown that AAV6 vectors are an excellent platform for donor delivery to primary hematopoietic stem cells, T cells and B cells. In order to further enhance this process, we systematically analyzed factors affecting lymphocyte transduction by AAV with the goal of maximizing gene addition while reducing cellular toxicity and AAV quantity required. Fetal bovine serum (FBS) inhibits transduction of human B cells by AAV6, and this is also true in several lymphocyte-like cell lines, where 10% FBS nearly completely blocks transduction at lower MOIs (10^3 and 10^4), though an MOI of 10^6 can overcome this inhibition. In K562 cells, inhibition at an MOI of 10^4 decreased linearly as FBS was decreased, and was abrogated at 0.01% FBS. In the absence of serum, MOI is the most important determinant of transduction levels. However, increasing the concentration of cells and AAV6 significantly increased transduction for a given MOI, with the greatest effects at higher MOIs. Consequently, across several MOIs, we achieved comparable transduction rates with 10-fold less AAV6 by simply concentrating the cells 10-100-fold. Performing AAV6 transductions with varying periods of serum deprivation revealed a biphasic pattern, with an initial burst of transduction occurring in the first hour, followed by linear increases out to 24 hours. Vector copy numbers tracked with transduction rates, suggesting that FBS inhibits AAV6 by blocking viral entry. Finally, when cells were incubated with AAV6 vectors and then electroporated, higher transduction rates were obtained if the cells were not washed prior to electroporation. Together, these findings suggest an optimized ZFN (zinc finger nuclease) plus AAV6 gene addition protocol, where cells are transduced at high concentrations in small volumes of media for one hour, diluted in electroporation solution without washing, electroporated with ZFN mRNA, and then transferred to serum-containing media. In primary human B cells, this protocol yielded gene addition of a GFP expression cassette at the CCR5 locus with around 100-fold less vector than was originally employed. The optimized protocol also reduced overall B cell cytotoxicity when compared to protocols using longer periods of serum starvation. Similarly, in $CD4^+$ T cells this protocol reduced cytotoxicity and edited equal percentages of cells at MOIs of both $3x10^3$ and 10^4, suggesting that AAV6 delivery was no longer limiting under these conditions. Thus, we have identified methods that improve the rates of gene editing and cell survival in human B and T lymphocytes, while significantly reducing the amount of AAV6 vectors necessary to achieve these outcomes.

803. Adapting CRISPR Gene Editing towards Mitochondrial DNA

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Mitochondria are unique organelles that harbor genetic content. Mitochondrial DNA (mtDNA) encodes 37 genes, including 13 subunits essential for the function of the electron transport chain. Mutations in mtDNA lead to devastating metabolic consequences causing neuromuscular disorders that have no effective cure. In the current revolution of genetic engineering by CRISPR, we propose to adapt the CRISPR Cas9 system towards manipulating mtDNA. We have targeted the *Stretococcal pyogenes* Cas9 endonuclease to the mitochondrial matrix and confirmed localization by immunofluorescence and Western blot. The delivery of the single guide RNA (sgRNA) to the mitochondria has posed significant challenges since mechanisms for the delivery of nucleic acids to the mitochondria are inefficient and not well defined in mammalian systems. We have utilized a series of RNA motifs identified from nuclear-encoded RNA found inside mitochondria in an attempt to shuttle the sgRNA into the mitochondrial matrix. A few RNA motifs showed enrichment of the sgRNA in the mitoplast fraction by quantitative PCR. Fluorescent labeling of the sgRNA also demonstrated partial localization with a mitochondrial marker. To determine the efficiency of SpCas9 endonuclease activity, the heavy strand promoter of mtDNA was selected as the target domain. Upon Cas9 mediated cleavage, the linearized mtDNA will be degraded given the limited repair machinery in mitochondria and that mtDNA clearance primarily operates as the main quality control pathway. We detected a modest depletion of wildtype mtDNA with two RNA motifs by quantitative PCR. Overall, we have been able to direct both components of the CRISPR system to mitochondria. Modifications to the RNA motifs or elucidation of RNA import mechanisms in mitochondria will help to improve the efficiency of sgRNA delivery to mitochondria and thereby motivate therapeutic use of CRISPR for mtDNA diseases.

804. Complete Gene Disruption by CRISPR-Based Multiallelic Knock-In Engages Alternative DNA Cleavage and RNA Splicing to Spare Essential Proteins

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Targeted gene disruption in diploid or aneuploid somatic cells has been a challenge hindering in-depth study on gene functions. Here, exemplified by hyperploid human cell line LO2, we demonstrated that simultaneous knock-in of dual reporters through CRISPR/Cas9-induced homology-independent DNA repair, permits one-step generation of cells carrying complete gene disruption of multiple alleles. Among six genes attempted, we easily generated stable single-cell clones carrying complete disruption of all four copies of *ULK1* gene, lacking all three copies of intact *FAT10* gene or devoid of intact *CtIP* gene at both alleles. While we fully confirmed the depletion of *ULK1* and *FAT10* transcripts as well as corresponding proteins, our study revealed robust flexibility of cellular mechanisms in generating in-frame aberrant *CtIP* transcripts after both *CtIP* alleles were disrupted. Through targeting different regions of *CtIP* gene with distinct donor designs, we consistently observed various chimeric *CtIP* transcripts that were in-frame and produced aberrant CtIP proteins. Sequence alignment showed that various alternative splicing at cryptic splice sites as well as alternative DNA cleavage at 4th nucleotide by CRISPR/Cas9 were engaged for preserving CtIP proteins. Altogether, our study demonstrates a universal dual-reporter knock-in approach for trackable gene disruption, which could be utilized in a wide range of applications. Meanwhile, these data suggest that more caution is needed when disrupting essential genes, where aberrant functional transcripts might be generated via flexible RNA splicing and alternative DNA cleavage.

805. Gene Editing of Human Hepatocytes by CRISPR-Cas9

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Numerous studies have shown that mouse hepatocytes can undergo efficient gene disruption by CRISPR-Cas9 mediated double-strand breaks. In addition, gene editing by homologous recombination has also been achieved. However, the efficiency of these processes in human hepatocytes have not been studied. Here, we report the use of rAAV vectors bearing CRISPR-cas9 endonuclease and donor homology for gene editing of human liver cells. We established a protocol for *ex vivo* transduction of human hepatocytes by rAAV prior to transplantation to immune-compromised *FRGN* mice, a strain that supports liver replacement by transplanted donor cells. We tested numerous rAAV serotypes and found that rAAV LK03 and DJ resulted in >90% transduction in short term culture *ex vivo* using GFP and TdTomato as reporter genes. AAV transduced hepatocytes were then trypsinized and transplanted into *FRGN* recipients, resulting in high levels (>80%) of liver repopulation. The use of *ex vivo* transduction of human hepatocytes allows for smaller doses of AAV to be used than would be needed *in vivo*. Additionally, *ex vivo* infection followed by transplantation allows for liver repopulation of hepatocytes that have been efficiently gene-edited *ex vivo*. This methodology should also allow precise gene repair by concurrent *ex vivo* co-infection of AAVs expressing Cas9 and the correction template. As a proof of principle, gene repair vectors capable of targeting the human albumin locus were designed and are being evaluated for gene correction efficiency this way. As expected, the human albumin locus was targeted and high levels (~70%) of indels were achieved in cells treated *ex vivo* with high MOI prior to transplantation. In addition, as an alternative to *ex vivo* infection, the albumin-targeting vectors are also being tested in chimeric mice repopulated with human hepatocytes *in vivo*.

806. Utilization of the RhD Locus as a Safe Harbor for Gene Therapy Applications

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Potential limitations associated with the use of integrating viral vectors for gene delivery has led to the exploration of new vehicles and strategies. The CRISPR-Cas9 system is a promising alternative. By exploiting the repair mechanism of homologous recombination, the CRISPR-Cas9 system can be employed to target nearly any active loci. In the context of gene addition, as opposed to gene correction, an inert genomic safe harbor is required. A genomic safe harbor is defined as a region in the genome that is able to provide adequate expression of newly integrated DNA without negatively impacting the host cell and/or organism. Genes previously investigated as potential safe harbors (AAVS1, CCR5, and ROSA26) have since been shown to have negative effects associated with the disruption of an endogenous gene. Notably, targeting CCR5 has resulted in increased risk for contraction of West Nile virus and Japanese encephalitis in humans (Sadelain et al. 2011). Moreover, the AAVS1 and ROSA26 sites are located near gene-rich regions with close proximity to active loci associated with malignancy. For these reasons, new locations and/or strategies need to be identified. We are proposing the active RhD locus of hematopoietic stem and progenitor CD34^{+} cells (HSPCs) for the safe integration of therapeutic transgenes. The RhD locus is an ideal location being that this locus is disrupted in a substantial number of individuals, and depending on the population, up to 43% of patients clinically classified as Rh-negative experience no phenotypic consequences (Perry et al. 2012). In addition, studies have shown K562 myeloid-erythroid leukemic cells designed to lack the Rh antigens can still significantly express the crucial RhAG glycoprotein, a necessary component of the Rh core complex involved in the transport of small molecules across the membrane of red blood cells (Cherif-Zahar et al. 1998). Therefore, we have performed initial proof of principle experiments delineating the potential use of the RhD locus as a novel safe harbor. Eight separate CRISPR guide RNAs have been designed and evaluated by TIDE analysis yielding a range of 5-60% targeting efficiency. We chose to continue our investigations with our most efficient guide RNA, hereon referred to as RhD^{-6}. Our guide RNA was given this name to reflect the location of complementation in relation to the RhD start codon. RhD^{-6} was subsequently used to demonstrate successful integration and sufficient expression of an exogenous GFP reporter gene upon delivery with a DNA donor template to facilitate homologous recombination. This was performed in both the K562 cell line and the immortalized human T lymphocyte Jurkat cell line. Targeting of the RhD locus was confirmed by DNA sequencing, while GFP expression was confirmed by flow cytometry. GFP expression was found to persist for upwards of

three weeks after nucleofection. In the same manner, a second clinically relevant therapeutic gene, FVIII, was able to be expressed from the RhD locus. These experiments set the stage for targeting of HSPCs, an attractive option for gene therapy applications in that their isolation protocols are well established, they demonstrate tremendous cellular output, and possess the ability to self-renew (thus providing long-term correction). Therefore, the RhD locus is a viable safe harbor that can be used to treat several hematological disorders.

807. Inhibiting Hepatitis B Virus Replication In Vivo with Viral DNA-Targeting Talens Comprising Obligate Heterodimers

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Chronic infection with hepatitis B virus (HBV) continues to be a significant cause of mortality and morbidity. Globally approximately 700,000 people die each year as a result of complications of the infection. Currently available therapies rarely eliminate the virus from chronic carriers, and the main reason for modest curative efficacy is absence of an effect on viral covalently closed circular DNA (cccDNA). This replication intermediate is highly stable and serves as transcription template for viral genes. Employing strategies that involve targeted gene editing of cccDNA offers the means to inactivate cccDNA permanently and effect a cure from chronic HBV infection. Previously we showed that engineered dimeric transcription activator-like effector nucleases (TALENs) are capable of mutating viral DNA specifically and significantly inhibit replication in vivo and in cultured cells. Although promising, a crucial requirement for translating this gene editing technology to clinical application is optimization of the specificity of the action of the TALENs. To improve precision of HBV-targeting nucleases, we engineered TALENs to include mutant FokI nuclease domains that only cleave duplex DNA when the paired variants are juxtaposed in a head-to-head orientation on the target DNA. Homodimeric subunits located at off-target site should be inactive. Initial evaluation in transfected liver-derived Huh7 and HepG2.2.15 cultured cells demonstrated good efficacy against HBV. The TALENs targeting the *surface* (*S*), *polymerase* (*P*) and *core* (*C*) viral open reading frames suppressed markers of replication by up to 80%, and targeted mutation was verified using CelI endonuclease. Efficacy of the obligate heterodimers compared favorably to that of the original TALENs. To assess efficacy in vivo, mice were hydrodynamically co-injected with an HBV replication competent plasmid and sequences encoding the TALENs. Serum concentrations of HBsAg were decreased by 65% and 92% by sequences encoding TALENs targeting *C* and *S* respectively. Similarly circulating viral particle equivalents were diminished by 99% and 62% by sequences encoding TALENs targeting *C* and *S* respectively. Efficacy of the first generation TALENs and obligate heterodimeric sequences was equivalent, and targeted mutation of viral sequences was demonstrated using the CelI assay. Normal serum activity of alanine transaminase indicated a lack of toxicity and suggested little off-target activity. Analysis using next generation sequencing is being initiated to evaluate on- and off-target mutagenesis in detail. The good efficacy of the obligate heterodimeric TALENs targeting HBV should be useful to improve specificity of the engineered nucleases.

808. Transcriptional and Position Effect Contributions to rAAV-Mediated Homologous Recombination

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Recombinant adeno-associated viral (AAV) vectors constitute one of the most promising tools for gene transfer. While the majority of AAV transduction events are episomal, our laboratory exploits the vector's ability to induce homologous recombination (HR) for targeted integration of therapeutic sequences into the host genome. Not only do nuclease-free, promoterless AAV-mediated gene targeting systems offer the permanence of gene transfer associated with integration, but a vector lacking a promoter reduces the chance for oncogene activation from off-target vector integration. AAV-mediated HR appears to be more efficient when targeting transcriptionally active loci. Our preliminary studies in mammalian cells have suggested up to a three-fold increase in HR rates at a single locus upon transcriptional induction. Yet, it is unclear if transcription itself or other factors that secondarily influence transcription, such as chromatin state, are directly linked to AAV-mediated HR. We therefore set out to establish how transcriptional rate and/or chromosomal position effects influence this type of HR. To do this, we developed a high-throughput strategy to map and quantify precision AAV-mediated HR genome wide by exploiting an engineered locus whose transcriptional rates are controlled by drug administration. To this end, we used lentiviral vectors to generate a pooled population of HAP1 cells each harboring a single-copy, doxycycline-inducible genomic site expressing eGFP. The population is subsequently infected with a library of AAV serotype DJ vectors designed to integrate mScarlet and a unique barcode that would allow for sorting of properly targeted cells based on gain of mScarlet and loss of eGFP expression. The genetic barcode is detectable in both the genomic DNA and in RNA transcripts originating from the doxycycline-inducible promoter, and it is being used to map the integration site and quantify the transcriptional rate at each targeted locus. To date, we have targeted a cell population comprising ~900 unique target sites with the barcoded rAAV library, with and without inducing transcription just prior to rAAV vector administration. Extracted barcodes have been used to quantify target site transcriptional rates by next generation sequencing. We will discuss these results in combination with those of the mapping method that is being used to identify the genomic coordinates of each targeted site. Ultimately, this analysis will allow us to (a) identify genomic loci at which HR preferentially occurred for mapping onto existing chromatin state maps, and (b) correlate the rate of AAV-mediated HR at each targeted locus with the target site transcriptional rate. Our studies will provide more insight into the mechanism of gene targeting by AAV, optimal target site selection, and potentially expand the use of AAV-mediated gene targeting for treating various genetic and acquired diseases.

809. Enhancing ZFN Expression Construct and Nuclease Activity Leads to Improvement of *In Vivo* Genome Editing Platform

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Sangamo Therapeutics currently has open three clinical trials in the first ever evaluation of *in vivo* genome editing. The goal of these trials is to utilize a ZFN-mediated genome editing strategy to permanently modify patient liver cells through insertion of a corrective transgene at the *Albumin* locus, following systemic AAV2/6 delivery. We are currently evaluating this approach using donor constructs encoding the genes that are defective in Hemophilia B, and mucopolysaccharidosis (MPS) types I and II. Therapeutic transgene insertion into the *Albumin* locus and co-opting its high transcriptional activity could potentially provide long-term expression of the corrective transgene in stably modified hepatocytes. Stable insertion also avoids any potential issues associated with non-integrating gene therapy approaches, which is particularly important in the liver-directed treatment of pediatric disease, as there is significant hepatic cellular division and potential for loss of episomal genomes during growth and development. This *in vivo* genome editing approach depends upon effective ZFN expression and nuclease activity in patient hepatocytes. The work described here highlights the potential for next-generation *in vivo* genome editing constructs, demonstrated by improvements in both ZFN expression and nuclease activity through the rational enhancement of a) the AAV-ZFN expression construct backbone and b) the coding ZFN sequences by modulating both the DNA-binding and nuclease domains of the ZFNs. Importantly, selective substitution of ZFN amino acid residues at the protein-DNA interface allows for increased ZFN activity, the ability to tolerate a SNP in the ZFN *Albumin* target site, and greatly increased specificity. These improvements were achieved while preserving the original ZFN target site, which allows use of the original transgene donor construct. These enhancements further highlight the advantages of using ZFNs as a tool for the correction of monogenic disease via *in vivo* genome editing.

810. Nucleolytic Behaviors of CRISPR-Cas9 In Vitro are Poor Predictors of In Vivo Activity for Gene Therapy

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CRISPR-Cas9 has evolved into a critical member of the gene editing toolbox since its introduction as a rapidly customizable nuclease. Although the customizable nature of Cas9 via gRNA modulation has afforded it a preference among rapidly developing gene therapy projects, it has seen a recent push for a deeper understanding of its targeting and nucleolytic activity *in vivo*. Development of *in vitro* screens for gRNA activity as well as off-target activity have provided a cheaper method for rapidly analyzing a large number of gRNAs prior to experimentation and validation *in vivo*. Herein we report on our analysis of a computationally predicted gRNA targeting one of the key genetic instigators of Chronic Granulomatous Disease, CYBB. This gRNA is designed in an effort to drive Cas9 induced cleavage of the gene resulting in integrative repair utilizing an exogenous gene fragment via Cas9 disruption as part of a developing *ex vivo* gene therapy. *In vitro* experimentation supported the preference for a gRNA targeting normal CYBB 676-C for effective targeting of the mutant gene due to its comparatively higher level, roughly 2-fold, of relative activity *in vitro* when compared to the gRNA designed to target the CYBB mutant 676-T. Subsequent *ex vivo* experiments have demonstrated that the *in vitro* behaviors of Cas9 in this instance were an improper indicator of behavior as it pertains to therapeutic application *ex vivo*. This suggests that our perceptions of *in vitro* gRNA associated targeting behaviors of the Cas9 nuclease are in need of further refinement and that *in vitro* evaluation of potential gRNAs should be further validated *in vivo* or with caveats detailed herein.

811. Engineering Epigenetic Memory Requires Both DNA and Histone Methylation

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Distinct epigenomic profiles of histone marks have been associated with gene expression, which is tightly regulated during development and important for cell identity. Acquired epigenetic alterations can lead to diseases, such as cancers, cardiovascular disease and mental disorders. It should now be possible to treat such disorders by altering the epigenetic information at specific loci. However, to avoid the need for lifelong expression of an exogenous modifier protein, we need to establish a persistent epigenetic state, as nature does. That way the activity of our modifier could be transient, but its effect on gene expression would be long-lasting. Towards this goal, we have created a dCas9-based toolbox to precisely target and correct epigenetic alterations. We investigated the activity of a broad collection of genomically targeted epigenetic regulators that could write epigenetic marks associated with a repressed chromatin state (G9A, SUV39H1, KRAB), DNMT3A as well as the first targetable versions of Ezh2 and Friend of GATA-1 (FOG1). Combinations of dCas9 fusions were evaluated at two disease-associated loci with the overarching goal to precisely engineer epigenetic memory. The most persistent gene repression required the action of both DNA methylatransferase and histone methyltransferases.

812. Non-Viral Mediated Delivery of CRISPR-Cas9 and Donor Templates in Hepatocytes to Treat Inherited Metabolic Diseases of the Liver

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Introduction: Inherited metabolic diseases (IMDs) are a large class of genetic disorders characterized by deficiency of essential enzymes

or other proteins critical for metabolism. Although individually rare, the collective occurrence of these diseases is roughly 1 in 800 births. The liver is a target tissue for many IMDs because of its critical role in the disease pathology. Treatments available for patients with IMDs are currently limited. The only curative therapy for IMDs of the liver is to replace the affected liver with a healthy liver from a donor. However, liver transplantation is associated with high mortality risks. Furthermore, complications, such as graft rejection, lifelong immunosuppression therapy, and organ shortages severely limit the use of liver transplantations for IMDs. Therapeutic gene editing has recently advanced to clinical trials for multiple IMDs of the liver. In these trials, ZFNs and donor templates are delivered to hepatocytes using adeno-associated viral vectors (AAV). While AAVs have high efficacy in preclinical studies, their potency in humans is not fully characterized. Further, AAV vectors have drawbacks, including the potential to integrate into the host genome causing mutagenesis, and prompt an immune response. Non-viral methods for delivering gene editing tools would avoid safety risks associated with AAV vectors as well as enable delivery of potent forms of these reagents in hepatocytes. The objective of our study is to optimize non-viral delivery of CRISPR-Cas9 nucleases and donor templates in hepatocytes as an alternative therapeutic strategy for IMDs of the liver. **Methods:** We optimized nucleofection procedures in primary murine hepatocytes and Hepa1-6 cells. The Hepa1-6 cell line derived from cancerous murine liver tissue was used as a model line to optimize delivery of Hpd-aiming CRISPR-Cas9 nucleases and donor templates. We compare the delivery of Cas9 as a plasmid DNA, mRNA, and purified protein along with sgRNA in Hepa1-6 cells and primary hepatocytes. In addition, we evaluate the effects of homology arm length and symmetry on the efficiency of gene editing in Hepa1-6 cells and primary hepatocytes. The efficiency of nucleofection was estimated by flow cytometry on cells nucleofected with eGFP plasmid DNA. The CRISPR-Cas9 on-target activity was evaluated using Tracking of Indels by Decomposition, and the off-target activity will be evaluated using the T7 Endonuclease assay. Targeted gene modification will be evaluated in cells nucleofected with Hpd-aiming CRISPR-Cas9 nucleases and single-stranded donor templates using a restriction fragment length polymorphism assay. **Results:** Our preliminary results indicate that CRISPR-Cas9 nucleases delivered as a plasmid DNA and mRNA have similar on-target activity in Hepa1-6 cells. We observed on-target indels of 22.3% for CRISPR-Cas9 plasmid DNA, and 22.1% for Cas9 mRNA delivered with sgRNA in Hepa1-6 cells. In on-going work, we will optimize delivery of Cas9 protein along with sgRNA as well as evaluate on- and off-target indels in Hepa1-6 cells and primary hepatocytes. Finally, we will optimize targeted gene modification in hepatocytes.

813. Optimization of the gRNA Vector Dose Enhances Long-Term Systemic AAV CRISPR Therapy in Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a chronic disease requiring long-lived whole body therapy. Recent studies have provided the proof-of-principle for treating DMD with adeno-associated virus (AAV) CRISPR gene repair therapy. However, long-term persistent therapy in both heart and skeletal muscle has not been demonstrated. To address this critical issue, we co-delivered a Cas9 and a gRNA AAV vector to 6-week-old mdx mice and evaluated mice at 18 months of age. Despite widespread dystrophin restoration in the heart, nominal restoration was detected in skeletal muscle. On western blot, restored dystrophin reached ~4% of the wild type level in the heart. Some ECG parameters were improved but cardiac histopathology and hemodynamics were not improved. Quantification of the vector genome copy number revealed a preferential loss of the gRNA vector. To test if preferential loss of the gRNA vector accounts for poor dystrophin restoration, we repeated the study with a 3-fold higher dose of the gRNA vector. At 18 months of the age, we detected similar levels of the Cas9 and the gRNA vector genome. Robust dystrophin expression was observed not only in the heart but also in skeletal muscle on immunostaining and western blot. Western blot of samples from the treated hearts showed dystrophin restoration was more than doubled compared to what we saw with the lower dose of gRNA vector. Treatment greatly reduced fibrosis in both skeletal muscle and the heart. Force measurement in the isolated extensor digitorum muscle showed significant enhancement of the specific twitch and tetanic forces and significant improvement in the eccentric contraction profile. Similar ECG improvement was observed as we saw with the lower gRNA dose. However, in contrast to the studies with low gRNA dose, cardiac catheter examination showed significant improvement of the heart hemodynamics. In summary, by increasing the dose of the gRNA vector, we achieved effective long-term CRISPR therapy for DMD. The optimization of Cas9 and gRNA expression may be applicable to CRISPR therapy for other diseases.

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814. Preclinical Modeling Highlights the Therapeutic Potential of the Adoptive Transplant of Gene Corrected T Cells in X-Linked Hyper-IgM Syndrome

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The X-linked hyper-IgM syndrome (HIGM1) is a primary immunodeficiency caused by mutations of the CD40 ligand gene (*CD40LG*). Physiologic expression of CD40L on activated CD4+ T cells provides essential signals for B cell activation and immunoglobulin class-switching, but its unregulated expression by randomly integrating gene therapy vectors has been shown to lead to lymphoproliferation and lymphoma. Therefore, we are developing a gene editing strategy aimed to correct the gene while preserving its physiologic expression control. Since in HIGM1, the genetic defect is not lethal to T cells, we aim to edit autologous T cells that could be used to provide immediate therapeutic benefit to patients by resolving pre-existing infections and a bridge to a definitive cure by employing Hematopoietic Stem/Progenitor Cells (HSPCs) transplant. We first optimized a CRISPR/Cas9-based protocol in human T cells and HSPCs by targeting the *AAVS1 locus*, and found that the delivery of a ribonucleoprotein (RNP) and an AAV6 donor exhibited ~40% of homology direct repair (HDR) integration, while preserving the T stem memory cells and the more primitive progenitors, respectively. We then screened and selected optimal CRISPR/Cas9 reagents targeting an upstream intron of the *CD40LG* to correct most of disease causing mutations with the same nuclease/donor set. After polyclonal stimulation, our best performing donor configuration restored CD40L surface expression and physiologic regulation on edited $CD4^+$ T cells from both healthy donors as well from a HIGM1 patient. The ability of corrected cells to provide contact-dependent helper function to B cell was confirmed by performing in-vitro class switching, proliferation and IgG secretion assays. In order to establish the therapeutic threshold and transplant conditions required to achieve immune reconstitution with corrected T cells, we took advantage of a suitable HIGM1 mouse model. We infused different low doses of wild type T cells into Cd40lg-/- mice pre-conditioned or not with different lymphodepleting regimens. Serial blood analyses showed long-term, stable T cell engraftment, with the highest rate obtained in mice infused after a mild chemotherapy treatment (cyclophosphamide). These mice partially rescued the antigen-specific IgG response after immunization with a thymus-dependent antigen (TNP-KLH) and showed the reconstitution of some germinal center B cells within splenic lymphoid follicles.

815. GMP-Compliant Non-Viral CRISPR-Mediated Process Correcting the Sickle Cell Disease (SCD) Mutation in SCD Patient $Cd34^+$ Cells Achieves 60% Wild Type Adult Hemoglobin Expression in Differentiated Erythrocytes

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The c.20A>T mutation of β-globin gene causes sickle cell disease (SCD). Allogeneic hematopoietic stem cell (HSC) transplantation can cure SCD, but most lack a suitable donor. Ex vivo gene therapy strategies, including lentiviral mediated gene transfer or endonuclease mediated BCL11a knockdown allowing fetal hemoglobin (Hb) induction, are currently under evaluation. Correction of the SCD mutation by non-viral gene editing of autologous HSCs would add an alternative strategy and permit endogenous gene expression at its natural regulatory locus and the beneficial reduction of the pathogenic sickle Hb production. Allogeneic transplantation has established that the therapeutic threshold for clinical benefit is ≥20% donor chimerism. We previously reported efficient correction of a monogenic "hotspot" mutation in the *CYBB* gene in X-linked chronic granulomatous disease (CGD) patient HSCs with a robust, scalable, cGMP, and regulatory compliant process (Sci Transl Med 2017) that we now apply to SCD. In initial studies using a B cell line (B-LCL) created from SCD patient and healthy volunteers' CD34+ HSCs, we developed a SCD mutation specific guide RNA, and a normal β-globin specific guide RNA (converse). The converse guide differed by only one nucleotide from the SCD mutation specific guide, where each guide could be used together with a single stranded DNA donor to effectively alter the wild type to SCD and the SCD to wild type, respectively (ASGCT 2017). At first, we optimized homology directed repair (HDR) at the SCD locus by integrating a HindIII enzyme site. We observed efficient site-specific insertion of the HindIII-marker in the B-LCL as evidenced by HindIII digestion of the PCR products (~50%), and targeted sequencing (~35% HDR and ~50% Indel). The optimized process was applied to correct SCD $CD34^+$ HSCs to achieve similar biallelic HDR rates for HindIII site insertion as well as gene correction from the SCD mutation to the normal β-globin sequence (up to ~35% correction and ~50% Indel). Interestingly, this correction was maintained during erythroid differentiation in culture. Among erythrocytes differentiated from corrected SCD $CD34^+$ cells *in vitro*, wild type adult Hb protein levels were above 60% as assayed by both reverse phase HPLC and Hb electrophoresis, and sickle Hb production decreased from 100% to 20% after correction. In summary, based on these in vitro correction rates confirmed by targeted sequencing, wild type adult Hb protein expression, and substantially decreased sickle Hb amounts, we are starting to evaluate engraftment of corrected SCD patient HSCs in immunodeficient mice. The high rate of engraftment

in immunodeficient mice of similarly corrected HSCs observed in our published CGD study puts these results observed for *in vitro* correction of SCD within the therapeutic window of reversing SCD.

816. Oncogene-Induced Senescence in Hematopoietic Progenitors Leads to Myeloid-Restricted Hematopoiesis, Chronic Inflammation and Histiocytosis

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Hematopoietic stem cell gene therapy (HSC-GT) has brought unprecedented benefits for diverse diseases; however, oncogenic activation and the consequent tumor development is a concerning side effect. Aberrant activation of the MAPK pathway by insertional mutagenesis is one of the most frequent culprits of tumorigenesis in previous studies from our lab in *Cdkn2a* deficient mice, particularly when vectors carried strong enhancers/promoters. However, in a wild-type genetic background aberrant MAPK pathway activation triggers oncogene-induced cellular senescence (OIS), which results in proliferation arrest and activation of a pro-inflammatory program, known as senescence-associated secretory phenotype (SASP). Although OIS is effective in contrasting cancer formation, SASP may contribute to chronic inflammation and exacerbate many diseases. For example, the oncogenic mutation $BRAF^{V600E}$ has been associated with senescence features in Langerhans cell histiocytosis (LCH), a lethal disease characterized by multi-organ accumulation of pro-inflammatory dendritic cells. To investigate the impact of OIS on hematopoiesis in HSC-GT settings we used bidirectional lentiviral vectors to stably express $BRAF^{V600E}$ or its wild-type counterpart (wtBRAF) and GFP in human hematopoietic stem and progenitors cells (HSPCs) and analyzed their effects *in vitro* and *in vivo* upon transplantation into immunocompromised mice. While mice transplanted with wtBRAF expressing HSCPs (n=12) had a normal hematopoietic reconstitution, all mice receiving $BRAF^{V600E}$-expressing HSPCs (n=16) died of bone marrow (BM) failure within 7 weeks from transplantation, even at low doses as 5000 mutated cells. Human engraftment was significantly lower than controls ($p<0.01$), in contrast to what usually occurs in mice transplanted with cancerous cells. HSPCs differentiated in dendritic cells that disseminated in various tissues with features of multisystem LCH and activated OIS, as indicated by β-galactosidase activity and up-regulation of cell cycle inhibitors ($p16^{Ink4a}$ and p21) and SASP (IL-1β, TNFα, IL-6, IL-8, CCL2, CCL4). Additional analyses indicated that $BRAF^{V600E}$ expression induces a proliferative burst followed by cell cycle arrest and myeloid differentiation after 1-2 weeks. Myeloid skewing *in vivo* was the result of hampered lymphoid development whereas colony assays showed dose-dependent impairment in erythroid colony formation. Finally, we observed induction of senescence also in $BRAF^{V600E}$-negative cells that we confirmed by culturing untransduced HSPCs with SASP-conditioned medium. In conclusion, we show that activation of OIS in HSPCs leads to histiocytosis characterized by cell cycle arrest, chronic inflammation and myeloid-restricted hematopoiesis. Along this model, oncogenic activation during HSC-GT does not necessary lead to tumor development but rather to senescence and altered differentiation. This paradoxical effect on hematopoiesis represents a paradigmatic example of antagonistic pleiotropy in which OIS restrains cancer while altering the microenvironment and eventually contributing to a lethal disease.

817. Using CRISPR/Cas9 Technology to Generate a Non-Human Primate Model of Clonal Hematopoiesis

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Recent population-based genomic studies have reported that clonally-expanded hematopoietic cells carrying somatic mutations are not only increasingly prevalent with age but are also linked to a higher risk of leukemia and cardiovascular disease. This phenomenon, termed clonal hematopoiesis (CH), is proposed to be a precursor to overt hematologic malignancy, with the most frequently mutated genes (top three: *DNMT3A*, *TET2*, *ASXL1*) encoding for epigenetic regulators. However, the relationships between these mutations, clonal expansion, and clinical outcomes are not well-understood because of challenges modeling them *in vitro* or in small animals. To address this, we performed autologous transplantation of rhesus macaque hematopoietic stem and progenitor cells (HSPCs) edited with CRISPR/Cas9 to create loss-of-function (LOF) mutations in the above genes. The first two macaques, ZK48 and ZI35, were transplanted with HSPCs edited at *AAVS1* (internal control) and *DNMT3A*. For ZK48, HSPCs were transduced with a gRNA-MSCV-GFP lentiviral vector (LV), then electroporated with Cas9 mRNA. To reduce LV-induced cytotoxicity, Cas9/gRNA ribonucleoprotein (RNP) complexes were used for ZI35. Both macaques demonstrated low (< 2%) mutation frequencies after 15 months (ZK48) or 14 months (ZI35) post-transplant (PT), and no expansion was observed. The third macaque, ZL26, received HSPCs edited with Cas9 RNP containing gRNA targeting *AAVS1* or a gRNA pool targeting *DNMT3A*, *ASXL1*, and *TET2*. Gradual but dramatic expansion of TET2-mutated clones was observed, with mutation frequency reaching >15% in granulocytes (Gr) by 19 months PT (Fig **1A**). Indels of these expanding TET2-edited clones are predicted to disrupt TET2 function. In contrast, there was no expansion of DNMT3A- or ASXL1-mutated clones. We next examined the TET2 mutation frequency of various mature hematopoietic lineages from peripheral blood (PB) and CD34+ cells from bone marrow (BM) (Fig **1B**). PB natural killer, B, and T cells were comparable to Gr. However, PB macrophages had a TET2 mutation frequency (~40%) almost thrice as high as PB Gr (~15%) by 19 months PT. TET2 mutation frequency was also more than twice as high in BM CD34+ cells (~18%) than in PB Gr (~8%) at 12 months PT (Fig **1C**). These

data suggest that TET2-mutated clones accumulate in the stem cell compartment and preferentially differentiate into macrophages. Additionally, compared to other transplanted macaques, ZL26's bone marrow exhibits hypercellularity and skewing toward the myeloid lineage. Furthermore, RNA-seq of wildtype and TET2-edited BM colony-forming units (CFUs) from ZL26 indicate that TET2-edited myeloid CFUs have a distinct gene expression profile, including higher expression of inflammatory cytokines like IL1β, which is of relevance to cardiovascular risk. A second macaque, ZL39, transplanted with TET2-targeted HSPCs also displays expansion specific to TET2-mutated clones. In summary, autologous transplantation of macaque HSPCs with CH-related mutations introduced by CRISPR/Cas9 resulted in expansion of only clones carrying TET2 LOF mutations, with no expansion of clones carrying DNMT3A or ASXL1 LOF mutations. We believe this model will provide a better understanding of CH's causes and consequences in a clinically relevant setting.

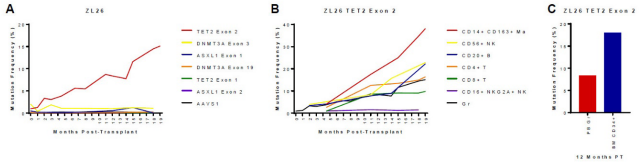

Figure 1 | Mutation Frequencies in Rhesus Macaque Model of Clonal Hematopoiesis
Targeted deep sequencing was performed on post-transplant granulocytes (**A**) and lineage-sorted cells (**B**, **C**) for ZL26. Ma, macrophages; NK, natural killer cells; B, B cells; T, T cells; Gr, granulocytes; PB, peripheral blood; BM, bone marrow; PT, post-transplant.

818. Targeted Gene Insertion for the Treatment of X-Linked Hyper-IgM Syndrome

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X-linked hyper-IgM syndrome (XHIM) is a primary immunodeficiency due to mutations in the CD40 ligand gene resulting in defects of immunoglobulin class switch recombination and somatic hypermutation. Previous gene therapy-based studies have investigated the use of retroviral vectors for delivery of CD40L cDNA, but abnormal lymphoproliferation was observed in mouse models due to uncontrolled expression of the gene, highlighting the need for alternative strategies. Here, we demonstrate the potential of both the TALEN and CRISPR/Cas9 editing platforms to allow homology directed repair-mediated integration of a normal copy of the CD40L cDNA delivered by Adeno-Associated Virus at the 5'UTR of the gene. Site-specific insertion of the donor sequence downstream of the endogenous CD40L enhancer and promoter maintains physiologic expression of CD40L while overriding all reported downstream mutations responsible for the disease. Gene modification can be achieved at high levels in cell lines and XHIM patient-derived T cells ranging from 20-30% with functional restoration of CD40L binding to its receptor (CD40) as measured by flow cytometry. Importantly, comparable rates of site-specific integration could be attained using either nuclease platform in human hematopoietic stem cells (HSC). Manipulated HSC engraft in immunodeficient mice at rates similar to mock-treated cells with clinically-relevant frequencies of site-specific gene insertion ranging from 2.5-4.5%, providing the foundation for a permanent curative therapy in XHIM patients.

819. Plerixafor Enables the Safe, Rapid, Efficient Mobilization of Haematopoietic Stem Cells in Sickle Cell Disease Patients

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Introduction: Sickle cell disease (SCD) is characterized by chronic anaemia and vaso-occlusive crises, which eventually lead to multi-organ damage and death. Implementation of the only curative treatment for SCD (hematopoietic stem cell transplantation) is limited by toxicity and the poor availability of HLA-compatible donors. A gene therapy approach based on the autologous transplantation of lentiviral-corrected hematopoietic stem and progenitor cells (HSPCs) was shown to be efficacious in one SCD patient. However, alterations in the bone marrow environment and the red blood cell properties hamper the harvesting and immunoselection of HSPCs from SCD patients' bone marrow. The use of Filgrastim to mobilize HSPCs was associated with severe adverse events in SCD patients. Thus, broader application of the gene therapy approach requires the development of alternative mobilization methods. **Methods:** We set up a Phase I/II clinical trial whose primary objective was to assess the safety of a single injection of Plerixafor in SCD patients. The secondary objective was to measure the efficiency of HSPC mobilization and isolation. Three SCD homozygous patients followed at Necker-Enfants Malades hospital and meeting our inclusion criteria were enrolled between May 2015 and January 2017. All suffered from severe SCD, with a history of acute chest syndrom and >2 vaso-occlusive crisis per year requiring hospitalization. P1 and P2 were transfused monthly because years of hydroxyurea treatment had proven to be ineffective. Hydroxyurea treatment was stopped in P3 3 months before mobilization. P3 was then transfused monthly until mobilization. Their granulocyte counts were $<10^4$ /µlitre. Furthermore, prior to plerixafor injection, patients received several erythrapheresis sessions, in order to decrease HbS level to below 30%. Each patient received a single, subcutaneous injection of Plerixafor (0.24 mg/kg). **Results**: No adverse events were observed. The patients exhibited a highly reproducible, very fast, and intense increase in peripheral blood $CD34^+$ cell count, which peaked at between 80 and 120 $CD34^+$/µlitre. Levels greater than 20 $CD34^+$/µlitre were maintained up for 12 to 24 hours and returned to normal pre-treatment values thereafter. Phenotypic analysis by flow cytometry revealed that the number of HSCs per 1000 $CD34^+$ cells was

>25 in Plerixafor-mobilized SCD samples and <5 in all other samples (bone marrow from healthy donors or SCD patients, filgastrim- and plerixafor-mobilized samples from healthy donors). By RNA-Seq, we observed that SCD samples were characterized by un upregulation of genes involved in inflammatory and immune responses, but also strong expression of HSC markers. Proof of stemness was further confirmed by transplantation into conditioned, immunodeficient mice, which led to stable human multilineage chimerism in both primary and secondary transplantation. **Conclusions:** Plerixafor can be safely used to mobilize HSPCs in SCD patients under well-defined clinical conditions. Importantly, the mobilized cells contained high numbers of HSCs, expressed high levels of HSC-related genes, and engrafted efficiently in immunodeficient mice. This finding opens new avenues for treatment approaches based on gene addition and genome editing.

820. Mechanism of Human Hematopoietic Stem Cell Loss during *Ex Vivo* Manipulation and Gene Transfer

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Hematopoietic stem cells (HSCs) are excellent targets for gene therapy (GT) to cure several inherited disorders. However GT requires *ex-vivo* manipulation and culture of HSCs, which largely results in their differentiation to hematopoietic progenitor cells. The tremendous loss of ex-vivo cultured HSCs with long-term repopulating potential (LTRP) has resulted in failure of numerous GT trials, and has necessitated very high doses of HSCs and pre-transplant conditioning for success. We have optimized a model of mobilized peripheral blood-derived adult human HSC (primarily used in GT) engraftment following lentivirus vectors (LV) or γ-retrovirus vectors (RV) in primary (6 mo) and secondary (1.5-3mo) NSG mice, for studying the LTRP of human CD34+ hematopoietic stem and progenitor cells (HSPCs) following *ex vivo* manipulation. We show that extended time of *ex vivo* culture (beyond 24 hours) of HSPCs itself results in substantial and progressive loss of LTRP, with the highest loss occurring with 3-4 days of culture. Furthermore, genetically manipulated cell progeny that was transduced with RV, showed a further tremendous loss of LTRP with loss of lymphoid potential; despite the fact that phenotypically, HSCs, MPP (multipotent progenitors), CMP (common myeloid progenitors), LMPP (Lymphoid-primed multi-potent progenitors) and MLP (multi-lymphoid progenitors) were comparably transduced (and LMPP/MLP showed higher transduction) to that by LV. More specifically, we show that non-cycling HSCs (genetically modified with either LV/CRISPR-Cas9) were more tolerant of genetic manipulation. However, cycling HSCs, when genetically manipulated with RV, or LV, lost LTRP, suggesting that a DNA double-strand break during HSC in active cell cycle triggered loss of LTRP. Mechanistically, we show increased activated DNA damage response (DDR) from increased p38MAPK, and CHK1 kinase signaling in cycling HSC, which results in accumulation of transduced/edited cycling HSCs in the G_2M phase of cell cycle, depleting HSC HIF1α. Targeting these pathways during LV/RV transduction reduced DDR, stabilized HIF1α and reversed the G2M accumulation, functionally, restoring the LTRP and multi-lineage potential of both transduced and untransduced HSPC, to levels seen in freshly isolated HSPC. Herein, we have identified fundamental mechanisms of loss of HSPC LTRP with genetic manipulation that can be targeted temporarily with small molecule inhibitors, without altering the mutational load of HSC (measured by whole exome sequencing). These studies have broad, important implications for the success of GT for HSC disorders.

821. Liver-Targeted Lentiviral Gene Therapy Mediates Long Term Factor Expression in Neonatal and Adult Hemophilia Mouse Models

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Lentiviral vectors (LV) are gaining prominence as a gene delivery vehicle due to their large capacity and ability to sustain transgene expression via integration. LV have been evaluated in numerous ex-vivo cell therapy clinical programs with promising efficacy and safety profiles. To assess the systemic gene delivery potential of LV, we have developed a liver-targeted LV system encoding human B-domain deleted Factor VIII (hBDDFVIII) or human factor IX (hFIX) and have evaluated this system in hemophilia mouse models. Codon optimized hBDDFVIII (coFVIII), unmodified hBDDFVIII (BDDFVIII) or codon optimized FIX (coFIX) were cloned into a hepatocyte specific LV expression system and corresponding third generation, VSVG pseudotyped LVs were administered into FVIII deficient (HemA) or FIX deficient (HemB) mice at neonatal or adult stage via intravenous injections. LV gene delivery enables stable integration of the transgene expression cassette into the genome of targeted cells. In the treated mice, persistent FVIII expression was observed post-LV treatment of 2 day old HemA mice throughout the 6-month study period; at 1.5E10 transducing units per kilogram (TU/kg) dose, while IV administered LV-BDDFVIII resulted in 5-10% of normal circulating FVIII, LV-coFVIII mediated up to 1000% of normal circulating FVIII, translating into a 100-fold improvement on FVIII expression. To determine the LV-coFVIII dose range could potentially mediates curative level of circulating FVIII in HemA mice, a dose deescalating study was performed and result shown that therapeutically beneficial levels of circulating FVIII was achieved at significantly lower LV doses, and this reduction in LV dose might be able to significantly lower any potential adverse effect associated with LV treatment. To determine if LV can effectively transduce both fast-growing and mature livers, HemB mice were treated with three dose levels of LV-coFIX at 2 days or 8 weeks of age. LV-coFIX was used here to avoid transgene mediated antibody responses observed against hBDDFVIII in adult HemA mice. Steady, persistent FIX expression was observed in all treatment groups, and furthermore LV treatment at neonatal and adult stage resulted in similar level of FIX expression when the same dose level of LV was administered. These results suggest that LV gene delivery approach might be effectively used in both pediatric and adult patient

populations. Gene integration is a unique property of LV which is lacking from most of the gene therapy programs that are currently under clinical investigation for hemophilia, and in conjunction with the rare incidence of pre-existing anti-VSVG-LV antibodies, our data support the further development of the LV system as a potential systemic gene delivery vehicle for hemophilia patients.

822. Biopotency and Efficacy of SHP648, a Next-Generation Fix Gene Therapy Vector

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Introduction. AAV-based gene therapy has shown potential clinical benefit in the treatment of hemophilia B. This is exemplified by a clinical phase 1/2 study (NCT01687608) testing the safety and efficacy of BAX 335 (AAV8.sc-TTR-FIXR338Lopt). BAX 335 is a self-complementary, recombinant AAV8 vector to deliver the codon-optimized human Factor IX (FIX) Padua transgene. In some patients, however, the expression of therapeutically relevant FIX levels was temporally limited. A root cause analysis indicated that exaggerated immunogenicity was the potential cause for decreased FIX expression. BAX 335 contains a high number of CpG dinucleotide motifs in the FIX coding sequence which have been proposed to contribute to increased immunogenicity by stimulating the innate immune system via the Toll-like Receptor 9 (TLR9) pathway. The rational design of a next generation vector SHP648 addresses these liabilities: The vector design included the replacement of the FIX coding sequence by a novel codon-optimized CpG- depleted FIX Padua transgene. Furthermore, the strength of the liver-specific transthyretin (TTR) promoter was increased by insertion of three liver-specific cis-regulatory elements (CRM8). This study examines BAX 335 and SHP648 dose-response relationships in FIX knockout mice, correlating increases in FIX activity levels and the respective hemostatic potential in a bleeding assay. **Methods.** Male FIX knockout mice (n=12/group) were administered single intravenous doses of SHP648 or BAX 355 (at $5x10^{10}$, $1x10^{11}$, $5x10^{11}$, or $1x10^{12}$ vector genome [vg]/kg, respectively) or 10 mL/kg buffer. FIX plasma activity levels were determined using a 1-stage clotting assay at days 7, 14, 28, 42, and 56. At the end of the observation period (day 56), the bleeding phenotype was assessed in a tail-tip bleeding assay. The viral transduction efficiency of liver tissue was analyzed by semiquantitative immunohistochemistry and quantitative real-time polymerase chain reaction. Preliminary safety assessments (clinical symptoms, mortality, and histopathology of selected organs) were also performed. **Results.** SHP648 and BAX 335 mediated FIX activity was detectable at all dose levels from day 7 to day 56 in plasma of FIX knockout mice. The treatments with SHP648 resulted in a dose-related increase in mean plasma FIX activity from 1 IU/mL ($5x10^{10}$ vg/kg cohort) to a supraphysiologic level of 37 IU/mL ($1x10^{12}$ vg/kg cohort) at day 56. SHP648 doses above $5x10^{10}$ vg/kg reduced blood loss significantly compared to buffer treated animals (p-value: <0.05) in a dose-dependent manner in the tail-tip bleeding assay. Treatments with BAX 335 resulted in mean FIX activity levels ranging from 0.4 to 4.5 IU/mL across all BAX 335 treatment groups. Significantly reduced blood loss was detectable only at BAX 355 dose levels above $1x10^{11}$ vg/kg in the tail-tip bleeding assay (p-value: <0.05). Transduction efficiencies of liver tissue correlated with the respective FIX activity data and bleed volumes. There were no in-life signs or deaths recorded in the treatment groups. **Conclusions.** Treatment of FIX knockout mice with the FIX gene therapy vectors SHP648 and BAX 335 did not reveal safety signals. SHP648 treatment groups showed higher FIX activity levels and improved control of induced bleeds when compared to BAX 335.

823. The Potential for Induction of ER Stress Response by an AAV5-Based Construct of B-Domain Deleted FVIII is Dependent on the Strength of the Hepatic Specific Promoter

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Hemophilia A (HA) is a bleeding disorder caused by deficiency of factor VIII (FVIII). Valoctocogene roxaparvovec (BMN 270) is an AAV5-based gene therapy vector that expresses a B-domain-deleted (BDD) human FVIII (hFVIII-SQ) under the control of hybrid liver specific promoter (HLP) and is currently being evaluated in severe HA patients. Malhotra *et al.* reported BDD hFVIII proteins accumulate in the endoplasmic reticulum (ER), activate an acute unfolded protein response (UPR) leading to ER stress, and induce apoptosis in mice hydrodynamically injected with plasmids encoding hFVIII genes. In addition, BDD hFVIII packaged in AAV8 capsids has been shown to transiently induce ER stress in mouse liver. To date, no evidence of liver dysfunction or ER stress had been observed in mice treated with valoctocogene roxaparvovec at doses that produce normal to supra-physiological levels of hFVIII-SQ. In instances where increased FVIII-SQ protein expression is desired, one approach is to use a stronger promoter. However, there is a possibility that a stronger promoter producing higher amounts of hFVIII-SQ protein could lead to ER stress. This study aimed to compare two AAV5 vectors with different promoter strengths (HLP vs 100ATGB, a stronger promoter) to drive hFVIII-SQ expression and study the effect of the stronger promoter on hepatic ER stress. Rag2-/- immunodeficient mice were dosed with vehicle, 6e12, 2e13 or 6e13 vg/kg of vectors and were euthanized 5 weeks after vector administration. Levels of liver hFVIIISQ DNA, RNA, and protein, as well as ER stress markers (spliced XBP1, CHOP, and molecular chaperones including GRP78), and levels of plasma hFVIII-SQ protein, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analyzed. There was a dose-dependent increase in the levels of liver hFVIII-SQ DNA and RNA for both vectors. The hFVIII-SQ RNA to DNA ratio was more than 6-fold higher for the 100ATGB vector than the HLP vector, confirming 100ATGB is a stronger promoter. At the 6e12 vg/kg dose, there was no detectable plasma hFVIII-SQ protein in mice treated with the HLP vector, while the mean plasma level of hFVIII-SQ protein was 71 ng/mL in mice treated with the 100ATGB vector. At the 2e13 vg/kg

dose, the 100ATGB vector produced between 2-3 fold higher plasma hFVIII-SQ protein concentration than the HLP vector, with mean levels of 140 and 58.2 ng/mL hFVIII-SQ protein, respectively. However, at the higher dose (6e13 vg/kg), the difference in plasma hFVIII-SQ protein concentration between the two vectors diminished to about 1.3 fold with mean hFVIII-SQ levels of 181 and 138 ng/mL for the 100ATGB and HLP vector, respectively. In mice treated with the 100ATGB vector, there was a dose-dependent decrease in the ratio of plasma hFVIII protein levels per unit of liver hFVIII-SQ RNA. This was accompanied by an increase in liver hFVIII-SQ protein accumulation and retention in the liver. Together, these data suggest hepatocytes may be reaching capacity to fold and secrete hFVIII-SQ protein in mice treated with a high dose of the 100ATGB vector. Indeed, at the 6e13 vg/kg 100ATGB vector dose, expression of several molecular chaperones (GRP78, PDIs, MANF, CRELD2, HYOU1) in the liver was induced, indicative of ER stress, but no evidence of apoptosis or liver injury was detected. In conclusion, a stronger promoter could produce higher levels of hFVIII-SQ protein at lower vector doses. However, at high vector dose, using a stronger promoter to drive the expression of hFVIII-SQ, a protein known to be difficult to fold and secrete, could lead to induction of ER stress.

824. Studying Clonal Dynamics of Hematopoietic Reconstitution and Vector-Induced Oncogenesis in Mouse Models of Hematopoietic Stem Cell-Gene Therapy by Longitudinal Vector Integration Site Analyses

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In hematopoietic stem and progenitor cell (HSPCs) gene therapy (GT), patients are transplanted with hundreds of millions of HSPCs harboring vector insertions semi-randomly distributed into their genome, many of which targeting oncogenes, tumor suppressors and other genes that modulate cell fitness. Despite the large number of insertions that could induce adverse events, in mouse models and clinical trials, leukemia occurs rarely. What is the behavior of cells harboring genotoxic insertion after transplant is unclear. To shed light on this issue, we performed a detailed clonal tracking of the hematopoietic reconstitution of mice transplanted with vector marked HSPCs in different conditions. Wild type (WT) mice were transplanted with HSPCs from tumor prone *Cdkn2a*$^{-/-}$ mice, which lack the barriers to proto-oncogene activations, and from WT animals, in which these barriers are fully active. WT and mutant HSPCs were transduced with a genotoxic Lentiviral Vector (LV) with active Long Terminal Repeats (LTR, LV.SF.LTR) or a neutral LV with Self-Inactivating (SIN, SIN.LV) LTRs. Mice transplanted with *Cdkn2a*$^{-/-}$ HSPCs transduced with LV.SF.LTR (N=25) developed tumors earlier than SIN.LV or mock controls (N=25 and 19 respectively, $p<0.0001$). On the other hand, no tumors developed in mice transplanted with WT HSPCs transduced with any vector (N=19 and 23). The hematopoietic reconstitution of monocytes, B and T cells was evaluated each month by cytofluorimetric analyses and by retrieval and analysis of vector insertion site analysis, as surrogate of clonal identity and abundance. No major differences in the early phases of hematopoietic reconstitution were observed among the different group of transplanted animals. Mice transplanted with *Cdkn2a*$^{-/-}$ HSPCs transduced with either vector showed major changes in blood composition only at later time points and before tumor onset. Unexpectedly, the type of vector used strongly impact on the hematopoietic reconstitution of mice transplanted with WT HSPCs. Indeed, while mice transplanted with mock or SIN.LV transduced HSPCs showed a similar progressive increase of the myeloid compartment, up to 45% of blood cells at 448 days after transplant, in LV.SF.LTR-treated mice the myeloid contribution was significantly higher (>60%). Analysis of >80.000 integration sites retrieved from the blood cells of transplanted mice showed a highly polyclonal repertoire in all lineages at early time-points and regardless of the vector type or genetic background used. At later time-points, mice transplanted with *Cdkn2a*$^{-/-}$ HSPCs showed a progressive reduction in clonality that was accelerated in LV.SF.LTR- treated mice. Furthermore, integration analyses performed in tumor-infiltrated tissues from mice transplanted with *Cdkn2a*$^{-/-}$ HSPCs transduced with the genotoxic LV revealed an enrichment of insertions in Braf. Mice transplanted with WT cells transduced with the genotoxic LV showed at later time points a marked reduction of clonality in the myeloid lineage with expansion of dominant clones. This phenotype was significantly less pronounced when a SIN.LV was used. Our results showed that the level of genotoxicity of the vector used strongly impact on the hematopoietic reconstitution of the transplanted mice, even in absence of tumor formation. Moreover, sporadic vector-mediated activation of proto-oncogenes could activate non-cell autonomous mechanisms of premature HSPCs ageing.

825. Genome Editing Using CRISPR/Cas9 and rAAV6 to Functionally Correct Wiskott-Aldrich Syndrome in Human HSPCs

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Wiskott-Aldrich syndrome (WAS) is a rare X-linked disease characterized by immunodeficiency with autoimmunity, a platelet disorder and an increased risk of hematologic malignancies. The pathogenic correlate are mutations in the WAS gene which lead to cellular defects in multiple hematopoietic lineages. Patients suffer from high morbidity and early mortality unless treated by allogeneic hematopoietic stem cell transplantation (HSCT), which is the standard of care but limited by donor availability, graft-versus-host disease (GvHD) and complications post-HSCT. Genome editing of autologous hematopoietic stem cells has the potential to correct all affected lineages and lead to long-term cure. We have developed an approach where the WAS cDNA is integrated seamlessly by homologous recombination at the transcription start site of the WAS gene in hematopoietic stem and progenitor cells (HSPCs) through creation of a DNA double-strand break (DSB) by a guided endonuclease that stimulates the endogenous repair machinery. To create the DSB we electroporate human HSPCs with Cas9 protein in a complex with a sgRNA and deliver the DNA donor template through recombinant adeno-associated virus 6

(rAAV6). We have identified an sgRNA that shows high nuclease activity and low toxicity. Bio-informatical analysis by *CRISPR Off-target Sites with Mismatches, Insertions, and Deletions* (COSMID) did not predict any off-target sites in regions of concern, which we are currently confirming experimentally. We have developed different constructs as DNA donor templates packaged in rAAV6 that can stimulate targeted integration of the WAS cDNA with or without a selection marker. When using the donor with co-expression of tNGFR, at least 20% of treated cells show expression of the marker and the NGFR$^+$ cells show WAS protein expression levels similar to wild type cells. When using donors without selection marker and evaluate targeted integration on the genomic level by droplet digital PCR (ddPCR), we measure frequencies of more than 50% of alleles. Using a high-fidelity Cas9 protein in combination with our targeting reagents lead to similar targeting efficiencies. Functional studies show that the threshold of a response to T cell receptor stimulation, which is shifted in cells with WAS knockout, is normalized in T cells expressing WAS from the integrated cDNA. In conclusion, we have developed an efficient and precise functional gene correction approach for Wiskott-Aldrich syndrome which we are continuing to evaluate in preclinical studies.

826. Recombinant Protein Guided Activation of HIV: a Means for Target Specific Purging of Provial Reservoirs

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The eradication of HIV is a collective goal that is hampered by viral persistence through the formation of provirus, burdening patients with life-long ART treatment. Several emerging modes of targeted therapeutics, ranging from chimeric antigen receptor (CAR) T-cell targeted killing of virus infected cells to targeted genomic editing and/or excision of latent provirus, may prove promising with regards to eradicating the spread of functional HIV. However, to functionally target HIV in the context of ART therapy and within the confines of the latent reservoir, a method to activate latent virus is needed. Currently, latency reactivating drugs are being tested, but suffer from variable efficacy in different latent HIV models as well as non-specific activation of host genes, which may cause unwanted toxic side-effects. A therapeutic compound that can specifically target and sustainably activate latent HIV provirus would prove transformative. Over the last decade much progress has been made towards the development of Zinc Finger Protein (ZFP) technology, which allows for recombinant proteins to be developed that can target specific genomic loci. We describe here the development and testing of an HIV specific ZFP protein fused to a VPR activation domain (ZFP-362-VPR), which potently activates HIV transcription in reporter and latency models. Furthermore, recombinant ZFN-362-VPR added directly to cells activates HIV in a target specific manner, and the effects were maintained in a range of HIV latency models. The advent of recombinant protein therapeutics may prove useful in targeting and purging reservoirs of HIV infected cells that could be used in conjunction with CAR T-cell and/or CRISPR based approaches.

827. Murine T Cells Modified with FVIII CAR and Foxp3 Alleviate Anti-Factor VIII Immune Responses

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The formation of immune responses towards factor VIII (FVIII; F8 in constructs) is a major obstacle for efficacious treatments to hemophilia A (Hem A) patients. According to our previous study, the FVIII primed polyclonal regulatory T cells (Tregs) had better suppressive function towards anti-FVIII immune responses compared to non-specific Tregs. However, the number of the antigen-specific Tregs was small in polyclonal Tregs. We adopted lentivirus (LV) systems to modify murine CD4$^+$ T cells with chimeric antigen receptor (CAR). The CAR construct was composed of human FVIII-specific scFv and signaling domains of CD28, 4-1BB, and CD3ζ (F8CAR). Fused by F2A peptide, murine Foxp3 cDNA was inserted at the 3'-end of the construct (F8CAR-mFoxp3) to provide transduced cells with regulatory characters. Transduction efficiency was tested in 293T cells and murine CD4$^+$ T isolated from the spleens of Hem A/BL6 mice. We demonstrated that the F8CAR-mFoxp3-LV produced high transduction efficiency in 293T cells, and the F8CAR expressed cells successfully bound to human FVIII protein. However, VSV-G-pseudotyped LV could not efficiently transduce murine CD4$^+$ T cells, even though the low number of transduced cells still exerted suppressive activity in a suppressive assay. Subsequently, we tested several envelop proteins and optimized the transduction protocol. As a result, Cocal-pseudotyped F8CAR-mFoxp3-LV consistently generated 25-35% transduction efficiency in CD4+ T cells. F8CAR-mFoxp3-LV transduced cells were then sorted and expanded with anti-CD3/CD28 dynabeads for 7 days. In a FVIII-specific suppressive assay, the transduced cells were co-cultured with effector T cells isolated from Hem A inhibitor mice. F8CAR-mFoxp3-LV transduced T cells exerted regulatory function and impeded proliferation of the effector T cells in the presence of APCs and FVIII protein. In addition, we generated F8CAR-LV transduced CD4$^+$ effector T cells which showed strong proliferative activity in the presence of APCs and FVIII. In a FVIII-specific CFSE assay, F8CAR-mFoxp3-LV transduced CD4$^+$ T cells significantly suppressed the proliferation of F8CAR-LV transduced CD4$^+$ effector T cells. To test the suppressive function of F8CAR-mFoxp3-LV transduced CD4$^+$ T cells *in vivo*, 0.4x10^6 F8CAR-mFoxp3 transduced T cells and non-specific polyclonal regulatory T cells were injected into the recipient mice, respectively, followed by hydrodynamic injection of FVIII plasmid a day later. F8CAR-mFoxp3 transduced T cells showed much stronger protective effect from high-titer inhibitory antibody production in mice compared to the non-specific polyclonal Tregs or mock control. The transferred cells were analyzed for F8CAR expression and Tregs affiliated biomarkers over time. Next, we will increase the dose of F8CAR-mFoxp3-LV transduced T cells in adoptive transfer experiments and evaluate their protective effect towards FVIII-specific inhibitor formation. In conclusion, we have successfully engineered murine CD4$^+$ CAR-T cells with Foxp3 and FVIII scFv gene expression that showed regulatory function towards FVIII-specific immune responses.

828. Induction of Fetal Hemoglobin Synthesis by CRISPR/Cas9-mediated Editing of the Human β-globin Locus

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Naturally occurring, large deletions in the β-globin locus result in hereditary persistence of fetal hemoglobin, a condition that mitigates the clinical severity of sickle-cell disease (SCD) and β-thalassemia. We designed a CRISPR/Cas9 strategy to disrupt a 13.6-kb genomic region encompassing the δ- and β-globin genes and a putative γ-δ intergenic fetal hemoglobin (HbF) silencer. Disruption of just the putative HbF silencer resulted in a mild increase in γ-globin expression, whereas deletion or inversion of a 13.6-kb region caused a robust re-activation of HbF synthesis in adult erythroblasts, associated with epigenetic modifications and changes in the chromatin contacts within the β-globin locus. In SCD patient-derived hematopoietic stem/progenitor cells (HSPCs), harvested from the bone marrow or mobilized in peripheral blood by Plerixafor administration, targeting of the 13.6-kb region using plasmid delivery of the CRISPR/Cas9 system resulted in high proportion of γ-globin expressing red blood cells and amelioration of the sickling cell phenotype. We then tested clinically relevant, transient and plasmid-free CRISPR/Cas9 delivery methods. Overall, ribonucleoprotein-based delivery exhibited a good balance between cytotoxicity and efficiency of genomic rearrangements, as compared to the other delivery systems, and resulted in up to 45% of deletion/inversion frequency and HbF up-regulation in erythroblasts derived from unselected edited HSPCs. Overall, this study validates the 13.6-kb region as a potential target to induce a β-to-γ reverse switching and provides clues for a potential genome editing approach for the treatment of β-hemoglobinopathies.

829. Applying Cas9/sgRNA-Mediated Targeted Genome Engineering to Generate a P47^{phox}-Deficient Induced Pluripotent Stem Cell Line for Gene Therapy Development

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Induced pluripotent stem cells (iPSC) are widely applied to model genetic disorders *in vitro*. Conventionally, disease-specific iPSC lines are generated via reprogramming of patient-derived cells. Depending on the cell source and reprogramming method, this process can be rather inefficient and the validation of pluripotency very time consuming. Recent advances in the Cas9/sgRNA technology allow fast and efficient genome editing of nearly every genomic position. We demonstrate that the Cas9/sgRNA system can be applied to introduce a patient-specific mutation into established, healthy iPSC for disease modeling of p47^{phox} deficiency, an autosomal-recessive form of chronic granulomatous disease (CGD), and to subsequently develop gene therapy. p47^{phox} deficiency is based on mutation of the *NCF1* gene and accounts for up to 25% of all CGD cases, which are characterized by defective phagocytes unable to kill encountered pathogens due to loss of NADPH oxidase activity. We introduced the most frequent mutation in p47^{phox} deficiency (c.75_76delGT, occurring in over 90% of patients) into healthy iPSC via homology-directed repair (HDR). Next generation sequencing revealed about 4% of both HDR and non-homologous end-joining in Cas9/sgRNA-treated samples. After identifying four p47^{phox}-deficient iPSC clones (out of 60 analyzed clones), detailed sequencing showed that the repair had most likely occurred via one of the two pseudogenes of *NCF1* due to high sequence homology (>98%) rather than via the applied donor template. To validate loss-of-function, the p47^{phox}-deficient iPSC clones were subjected to an embryoid body-based myeloid differentiation protocol, which yielded 53.1% ± 19.24% CD66b$^+$ granulocytes within the CD11b$^+$/CD16$^+$ population. Using the dihydrorhodamine assay, we demonstrate that differentiated cells completely lack NADPH oxidase activity. To provide a perspective for site-specific correction of *NCF1* mutations without off-target cleavage in the pseudogenes, whose transcripts might play a role as non-coding RNAs in regulatory processes, we developed a gene correction approach that targets only the functional gene by exploiting the presence of single nucleotide polymorphisms in the pseudogenes. Delivery of Cas9/sgRNA and a donor template carrying a minigene to restore p47^{phox} expression achieved targeted insertion of the donor into the *NCF1* locus in six out of 28 analyzed clones. In summary, we successfully generated a patient-specific p47^{phox}-deficient iPSC line using Cas9-mediated HDR. By introducing a DNA double-strand break into *NCF1*, we mimicked gene conversion as it occurs in the patients' genomes during disease development, copying parts of the pseudogenes that carry the disease-causing deletion into the functional gene. Therefore, the genetic correction of *NCF1* mutations using targeted genome editing for clinical application has to be carefully evaluated regarding both efficiency and safety.

830. Correcting Hemophilia Using *In Vivo* Therapeutic Protein Production by an Afibromer™ Encapsulated Gene Modified Allogeneic Cell

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Current hemophilia therapies require frequent protein infusions yet are unable to adequately address long-term sequelae due to compliance, troughs and immunogenicity. To overcome these drawbacks alternative delivery methods such as gene therapy are being investigated. The aim of this study is to determine whether the chronic delivery of blood clotting factors by implantation of gene modified cells producing factor is a more controlled viable alternative with significant upside for patients due to elimination of protein administration and better outcomes due to stable production levels. Proprietary allogeneic human cell line engineered to stably express high levels of hFIX or FVIII and placed in Afibromer™ capsules maintain long term cell viability and stable protein production. The encapsulation inhibits immune cell rejection and the novel chemistry prevents the foreign body and fibrotic responses that have historically plagued such modalities. A time course experiment revealed a rapid secretion of hFIX or FVIII through the capsules. Upon murine implantation human hemophilia factors are detected in the plasma at therapeutically relevant levels and are able to reverse bleeding pathology in factor deficient mice. The protein blood levels (PK) are dose dependent. Dosing is achieved by establishing the volume of capsules implanted. Taken together, these data confirm that stable clotting factors secretion by encapsulated engineered cells create a viable alternative to protein delivery or gene therapy with several important advantages such as ability to treat pediatrics, redose patients and more carefully control dosing. This platform is amenable to treatment of chronic diseases beyond hemophilia where long term constant protein production is required.

831. Cell Models of Hemoglobinopathy for Screening Therapeutic Approaches

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Mutations in the beta-globin gene (*HBB*) give rise to a group of disorders known as hemoglobinopathies. The most common of these are β-thalassemia and sickle cell disease (SCD) which affect millions of patients worldwide. The only curative therapy available to hemoglobinopathy patients is an allogeneic hematopoietic stem cell transplantation (HSCT), however only ~15% of patients have a matched sibling donor. It has been demonstrated that elevated levels of fetal hemoglobin (HbF), such as those in persons with hereditary persistence of fetal hemoglobin (HPFH), can have protective effects in hemoglobinopathy patients. Expression of HbF can compensate for the primary beta-globin deficiency, often resulting in attenuated symptoms or curative clinical outcomes. In SCD patients, hydroxyurea therapy is commonly used to induce HbF. However, less than 50% of patients respond, the effectiveness of the therapy diminishes over time and the mechanism of action is poorly understood. Genome-wide association studies have identified BCL11A as a modifier gene which suppresses HbF expression and has made it a target of gene-editing approaches. To facilitate the identification of small molecules or gene-editing approaches to treat SCD, we have introduced the sickle mutation into *HBB* of the recently described HUDEP-2 erythroid progenitor cell line to generate sickle HUDEP (S-HUDEP) cells using CRISPR/Cas9 cased genome editing. Upon erythroid differentiation and culture in a hypoxic environment, S-HUDEP cells undergo sickling making this a useful model for studying the effect of targeted mutations or small molecules on the sickle phenotype. We also introduced HPFH mutations into HUDEP cells and found that these HPFH-HUDEP cells express HbF with over 80% of cells positive for HbF expression. Testing of these HPFH mutations in S-HUDEP cells is underway. We also introduced modifier mutations not associated with globin switching into genes associated with rescue of the sickle phenotype. These mutant clones will undergo differentiation to observe their effect on cell sickling. Genes identified as true modifiers of the disease phenotype will be targeted in a selective drug screen to identify small molecules that rescue the sickling phenotype. In summary, we have used CRISPR/Cas9 to generate erythroid progenitor lines containing the sickle mutation. This isogenic mutant line will allow for elucidation of direct genotype-to-phenotype relationships between putative HPFH and modifier mutations in *HBB* and globin expression and sickling physiology of red blood cells respectively. This system will inform gene-editing approaches that target HbF reactivation and will accelerate drug discovery for sickle cell anemia.

832. Leukocyte Adhesion Deficiency-I: a Comprehensive Review of Published Cases

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Introduction: LAD-I is a rare leukocyte adhesion disorder caused by *ITGB2* mutations resulting in CD18 deficiency. Severe LAD-I (<2% of normal neutrophil [PMN] CD18 levels) is characterized by recurrent serious infections and early mortality unless treated by allogeneic hematopoietic stem cell transplant (HSCT). Mortality in severe LAD-I was 75% by age 2 in an initial 1988 retrospective study. Moderate LAD-I (2-30% of PMN CD18) is more indolent,

with recurrent skin and mucosal infections. We sought an updated understanding of severe LAD-I including prognosis in the absence of HSCT, HSCT outcomes and association of CD18 expression with clinical features. **Methods:** We created a database of published LAD-I cases via Pubmed searches and reference review. **Results:** 323 LAD-I cases were reported between 1975-2017 in 107 publications with the highest reporting from Iran (n=65), USA (n=50), and India (n=45). 113 pts were reported as severe LAD-I, 63 moderate and 147 not classified. PMN CD18 expression was reported for n=265 and was <2% in 51%. For pts with CD18<2%, median presentation was age 1m (range 0.03-18m); for CD18 ≥2%, median presentation was age 6m (0.03-192m). The most frequent infections in pts with CD18 <2% were respiratory tract (39%), sepsis (29%) and otitis media (27%) and for pts with CD18 ≥2% they were periodontal (52%), otitis media (36%) and sepsis (25%). Skin infections and ulcers were noted in >10%. Umbilical complications were more frequent in severe LAD-I (84% vs 58%). Median WBC was 45 x 10^9/L; range 10 - 150 x 10^9/L). There were limited correlations between CD18 expression and WBC and between CD18 and CD11 expression. Mutation analyses indicated >20 *ITGB2* gene locations; Exon 5, 6 and 7 mutations accounted for 44% of specified cases. We sought to understand whether prognosis for severe LAD-I in the absence of HSCT is similar to the initially-reported 25% survival to age 2. There were 66 severe LAD-I cases (per investigator assessment or CD18 <2%) for whom survival to 2 years was reported, 40 of whom died prior to age 2 (61% mortality). Mortality was similar (56%) for cases reported since 2000. The majority of pts with CD18 >4% survived to adulthood. HSCT conferred phenotypic correction in 83% of pts with HLA-matched sibling donors. Mortality was 19% (11% for matched sibling, 32% for haploidentical HSCT). **Conclusions:** Severe LAD-I remains a life-threatening condition with limited 2-year survival in the absence of allogeneic HSCT. Umbilical complications, granulocytosis, respiratory tract, ear, and systemic infections are common. HSCT is potentially curative with higher transplant-mortality in haploidentical recipients. Diverse mutations result in LAD-I; genetic evaluation may aid diagnosis. Rapid identification of potential LAD-I (unusual or severe infections in infancy, granulocytosis and umbilical complications) is essential to enable referral to centers of expertise.

833. Towards the Gene Therapy Clinical Trial for Pyrivate Kinase Deficiency

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Pyruvate kinase deficiency (PKD) is an autosomal recessive disorder caused by mutations in the PKLR gene. PKD is the most common erythroid inherited enzymatic defect causing chronic nonspherocytic hemolytic anemia and is associated with reticulocytosis, splenomegaly and hepatic iron overload. In severely affected patients PKD can be life-threatening. Splenectomy confers reduced transfusion-dependence in many patients, but 10-15% of PKD patients remain transfusion-dependent despite splenectomy, which confers increased lifelong susceptibility to systemic infections. Allogeneic bone marrow transplant has been curative in a small number of severely affected patients to date, but it has been employed infrequently. Preclinical gene therapy studies conducted in pyruvate kinase deficient mice have shown the safety and the efficacy of the PGK-coRPK-Wpre therapeutic lentiviral vector (CPcoRPKW-17) that has been granted orphan drug designation by the European Medicine Agency (EU/3/14/1330) and the US Food and Drug Administration (FDA#DRU-2016-5168). In order to develop a gene therapy clinical trial for PKD we have developed an optimized GMP-grade lentiviral vector production according to manufacturing process of the CMO VIVEbioTECH (www.vivebiotech.com). Using these GMP-grade lentiviral vectors we have defined the minimum number of transduced cells capable of correcting the hemolytic anemia (0.3-0.4 VCN/cell) in a mouse model of PKD. In accordance with these results, mouse bone marrow competition experiments showed that 30% of donor chimerism from wild type healthy donors was sufficient to restore correct the disease. Biodistribution studies have been also conducted in mice, in whom 5-fold vector integration per cell genome above the proposed clinical dose were transplanted in lethally irradiated recipients without evidence of medicinal product toxicity, and without reactivation or presence of the lentiviral vector in any non-hematopoietic organs. Optimization of human cellular transduction conditions, including the addition of transduction enhancers, enabled transduction in up to 90% colony forming cells tested in semisolid cultures, with up to 3 VCN/cell. Analysis of 14-day liquid cultures showed values between 1 and 3 VCN/cell. The addition of transduction enhancers did not alter cell viability or the engraftment capacity of the transduced human hematopoietic progenitors when transplanted in immunodeficient mice. The documentation required for the approval of the gene therapy trial will be presented. Study activation and initial patient recruitment are anticipated during the coming year.

Metabolic, Storage, Endocrine, Liver and Gastrointestinal Diseases III

834. The First Viable Mouse Model of *cblC* Type Combined Methylmalonic Acidemia and Homocysteinemia: A Single Dose of AAV Rescues Neonatal Lethality and is Comparable to Chronic B12 Injection Therapy

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Combined methylmalonic acidemia and homocysteinemia, *cblC* type (*cblC*), is the most common inborn error of intracellular cobalamin metabolism caused by mutations in the *MMACHC* gene. MMACHC transports and processes cobalamin into its two active cofactors, 5´-adenosylcobalamin and methylcobalamin, necessary for the enzymatic reactions of methylmalonyl-CoA mutase and methionine

synthase, respectively. Disease manifestations can be severe, especially in those with early onset disease, and include growth failure, anemia, heart defects, neurocognitive impairment and progressive blindness. We used genome editing to create pathogenic *Mmachc* mutations, and further studied two alleles: c.163_164delAC p.Pro56Cysfs*4 (Δ2) and c.162_164delCAC p.Ser54_Thr55delinsArg (Δ3). A decreased number of homozygous mutant pups were noted at birth ($p<0.0005$). Dissection of the pregnant dams at E18.5 showed that *Mmachc*$^{\Delta 3/\Delta 3}$ embryos were present in predicted ratios but had decreased crown rump length and weight ($p=0.0016$), suggesting intrauterine growth retardation. The median survival of the mutant mice was less than 7 days, with complete lethality by 1 month (Δ2/Δ2 n=13; Δ3/Δ3 n=42 $p<0.0001$). At 2 weeks, the weights of *Mmachc*$^{\Delta 3/\Delta 3}$ mice (n=9) were reduced relative to controls, with mutants 35% smaller than their littermates (n=94; $p<0.0001$). Mutants (Δ2/Δ2 n=4, Δ3/Δ3 n=6) displayed the biochemical features of *cblC*, with significantly elevated plasma methylmalonic acid, homocysteine, cystathionine and decreased methionine compared to wild type controls (n=18) ($p<0.02$). Pathological examination revealed hydrocephalus and hypoplasia of the corpus callosum, adrenal cortex, and testes in *Mmachc*$^{\Delta 3/\Delta 3}$ mutants with variable ocular pathology. To explore systemic gene therapy as a treatment for cblC, we generated two AAVs: rAAVrh10-CBA-mMmachc and rAAV9-CBA-hMMACHC that were delivered by a single neonatal intrahepatic injection (1 x 1011 GC/pup) and compared with weekly hydroxocobalamin (OHCbl) injections. Mmachc$^{\Delta 3/\Delta 3}$ mice treated with AAVrh10 (n=9), AAV9 (n=11) and OHCbl (n=9) displayed dramatically improved clinical appearance with increased survival ($p<0.0001$), with the oldest treated mutants living beyond 1 year. We also observed improved growth ($p<0.05$) and reduced plasma methylmalonic acid ($p=0.03$) at 2 weeks following AAV9 treatment. At 5 months, AAV genomes were detected in the heart (0.35 AAV GC/haploid genome), liver (0.08) and at lower levels in the lung, muscle, brain and spinal cord (n=5). In summary, this mouse model recapitulates the disease manifestations seen in humans with cblC including intrauterine growth retardation, decreased survival, poor growth and metabolic abnormalities. A single neonatal injection of an AAV vector, expressing either the mouse or human MMACHC gene, produces equivalent metabolic and phenotypic effects as chronic, injectable OHCbl treatment in the Mmachc mutant mice. We therefore suggest that AAV gene therapy could offer a promising new therapeutic approach to treat the systemic, and perhaps ocular, manifestations experienced by patients with this devastating metabolic disorder.

835. Toward a Hematopoietic Stem Cell Gene Therapy of Hemophilia A

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While hemophilia A gene therapy based on rAAV-mediated liver transduction and expression of factor VIII (FVIII) from hepatocytes has shown first promising clinical outcomes, it also has a number of disadvantages including the gradual loss of transgene expression due to hepatocyte turnover, induction of FVIII inhibitor antibodies, the high cost of rAAV vector manufacturing, and the risk of rAAV genotoxicity due to preferential integration into genes, specifically in patients with underlying chronic hepatitis virus infection, which is still a large fraction of hemophilia patients. These problems could potentially be overcome by in vivo hematopoietic stem cell (HSC) gene transfer using integrating HDAd5/35++ vectors for FVIII expression preferentially in peripheral red blood cells. Our proposed approach has the potential for live-long therapeutic correction after a single intravenous intervention. The enormous amplification of gene modified HSCs upon differentiation into red blood cells and the high-efficiency protein synthesis machinery of these cells creates a basis for FVIII production at curative levels. Furthermore, the genetic modification of only a fraction of HSCs results in tolerance against the transgene product most likely through tolerogenic T-cells in the thymus. We have developed a new approach for *in vivo* gene delivery into HSCs that does not require myeloablation and HSC transplantation. It involves injections of G-CSF/AMD3100 to mobilize HSCs from the bone marrow into the peripheral blood stream and the intravenous injection of an integrating, helper-dependent adenovirus (HDAd5/35++) vector system. HDAD5/35++ vectors target CD46, a receptor that is expressed on primitive HSCs. Transgene integration is achieved (in a random pattern) using a hyperactive Sleeping Beauty transposase (SB100x). We demonstrated in adequate mouse models, using GFP as a transgene, that primitive HSCs transduced in the periphery home back to the bone marrow where they persist and stably express GFP long-term. To achieve high-level (>80%) transgene marking in differentiated peripheral blood cells, we combined our in vivo HSC transduction approach with in vivo selection of transduced HSCs using the mgtm^{P140K} mutant gene and low dose O^{6}BG/BCNU treatment. Here we generated an integrating HDAd5/35++ vector containing the gene for ET3, an optimized human FVIII variant, under control of a 5kb version of the β-globin locus control region (LCR). The LCR mediated high-level, erythroid-specific, position-independent expression FVIII in in vitro differentiated human erythroid cells, but also low-level expression in lymphoid progenitor cells. We performed an *in vivo* HSC transduction study with the HDAd-FVIII vector in mobilized hCD46tg mice. O^{6}BG/BCNU in vivo selection will be started in February and serum FVIII levels and activity in mice, with and without immunosuppression, will be reported.

836. In Utero Genome Base Editing Cures Hereditary Tyrosinemia in a Mouse Model

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Introduction: Hereditary tyrosinemia type I (HT-I) is a lethal congenital liver disease caused by a deficiency in fumarylacetoacetate hydrolase (Fah), an enzyme in the tyrosine degradation pathway, that results in the accumulation of toxic metabolites leading to death in early infancy. The only treatment aside from liver transplantation is the drug NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione), which inhibits an upstream enzyme in the pathway (hydroxyphenylpyruvate dioxygenase (Hpd)) thereby preventing the accumulation of toxic metabolites and rendering the phenotype benign. Genome editing using CRISPR-Cas9 possesses great potential to treat

many monogenic disorders. Many of these disorders, such as HT-1, can be prenatally diagnosed and result in significant morbidity and/or mortality before or shortly after birth. Thus, the prenatal application of CRISPR-Cas9 genome editing has the potential to take advantage of the normal developmental properties of the fetus while treating a disease before its onset. Although promising, traditional CRISPR-Cas9 genome editing results in double-strand breaks (DSBs) which carry a high risk of unwanted mutagenesis. As an alternative, base editors can alter a single nucleotide in a sequence-specific fashion without the need for DSBs. In the current study, we employ CRISPR-Cas9 base editing to introduce a nonsense mutation in the *Hpd* gene in HT-1 mice *in utero*, rendering treated fetuses free of disease at birth. **Methods:** Intravenous injections of adenoviral vectors carrying a base editor designed to introduce a site-specific nonsense mutation into the *Hpd* gene (BE3-HPD) were performed in embryonic day 16 (E16) fetuses homozygous for the mutation causing HT-I ($Fah^{-/-}$). Control injections were also performed at E16 in $Fah^{-/-}$ fetuses using an unguided base editor (BE3-pA). NTBC treatment was withdrawn on the first postnatal day and mice were harvested at the time of death or at one month of age (P30) if they survived. Editing was quantified by both deep sequencing of liver DNA and liver immunohistochemistry staining for the Hpd protein. Phenotypic analysis included survival, weight change, and serum liver function testing (AST, ALT, and total bilirubin). **Results:** $Fah^{-/-}$ mice injected with BE3-HPD (n=13) demonstrated superior weight gain compared to control $Fah^{-/-}$ mice on NTBC treatment (n=7) ($p<0.01$ at P30) and were thriving at one month of age. In contrast, all $Fah^{-/-}$ mice injected with BE3-pA (n=18) lost weight and died within 3 weeks of birth. BE3-HPD treated $Fah^{-/-}$ mice also had normal liver function tests comparable to control $Fah^{-/-}$ mice on NTBC treatment, while $Fah^{-/-}$ mice injected with BE3-pA demonstrated significant liver damage prior to death (AST: 68 vs. 78 vs. 287, $p<0.0001$; ALT: 49 vs. 55 vs. 171, $p<0.0001$; total bilirubin: 0.1 vs. 0.1 vs. 3.2, $p<0.0001$). Deep sequencing demonstrated an average editing efficiency of 39% (range: 16-66%), and immunohistochemistry revealed approximately 90% absence of Hpd protein expression at one month of age (Figure 1). **Conclusion:** The novel *in utero* delivery of a CRISPR-Cas9 base editor yields efficient genome editing resulting in cure at birth of a lethal congenital disorder in a mouse model. This study provides proof-of-concept that *in utero* genome editing is a highly promising approach for the treatment of monogenic metabolic diseases.

837. Counteraction of Obesity and Insulin Resistance by Liver-Specific AAV-Mediated BMP7 Overexpression

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Type 2 diabetes (T2D) and obesity are very strongly associated and are a major health problem because of their alarmingly growing prevalence worldwide. Obesity is largely due to a sustained imbalance between energy intake and expenditure. Therefore, therapeutic approaches targeting metabolic rate may hold great potential for the future treatment of T2D and obesity. In this regard, bone morphogenic protein 7 (BMP7) has been reported to be a potent inducer of non-shivering thermogenesis via induction of brown adipogenesis, activation of uncoupling activity of brown adipose tissue (BAT) and browning of white adipose tissue (WAT). The intravenous administration of AAV8 vectors encoding a murine optimized BMP7 coding sequence under the control of a liver-specific promoter in high fat diet (HFD)-fed obese mice resulted in specific overexpression of BMP7 in the liver and very high levels of the factor in circulation. Noticeably, AAV8-BMP7-treated mice normalized their body weight and reversed insulin resistance. This was parallel to a marked reduction in food intake and enhancement of energy expenditure. AAV8-BMP7-treated mice also showed normalization of the weight of white fat depots and of the liver as well as reversion of HFD-associated WAT hypertrophy and inflammation and decreased hepatic steatosis and inflammation. Moreover, the treatment with AAV8-BMP7 vectors also increased browning of the subcutaneous WAT as evidenced by the higher number of multilocular adipocytes and increased expression of thermogenic markers such as UCP1, Cidea and Pparg1a. Altogether, these results underscore the potential of liver-specific AAV-BMP7-mediated gene therapy to treat T2D and obesity.

838. A Single Intravenous Injection of an AAV-PHP.B Vector Encoding Human Acid α-Glucosidase Corrects Both Muscle and Brain Defects in Murine Pompe Disease

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Background: Deficiency of acid α-glucosidase (GAA) in Pompe disease (glycogen storage disease type II) results in massive accumulation of lysosomal glycogen in all tissues. The disease manifests as severe cardiac and skeletal muscle myopathy, and central nerve system (CNS) defects have recently been recognized in both human patients and Pompe disease (GAA-KO) mice. Enzyme replacement therapy with recombinant human GAA reverses cardiac abnormalities but has little effect on skeletal muscle and the brain. AAV-mediated systemic gene therapy has shown great promise in preclinical studies for improving

correction of skeletal muscle but not the brain due to the presence of the blood-brain-barrier, while intrathecal injection of an AAV vector expressing human GAA (hGAA) into GAA-KO mice corrected glycogen storage mainly in the CNS. The newly developed AAV-PHP.B vector has shown high efficiency in transducing both the CNS and muscle tissues in mice. **Methods:** An AAV vector (AAV-CB-GAA) that encodes hGAA under the control of the universally active CMV enhancer/chicken β-actin (CB) promoter was packaged as AAV-PHP.B (capsid plasmid was kindly provided by Dr. Benjamin Deverman of California Institute of Technology). Two-week-old GAA-KO mice were intravenously injected with the AAV-PHP.B vector at a dose of $5x10^{12}$ vg/kg. Age-matched untreated (UT) GAA-KO mice were used as controls. Improvement of neurologic and neuromuscular function was evaluated by behavioral tests including cylinder test, beam walking, footprint, and rota-rod to assess motor coordination and balance; von Frey test to detect the sensory defect; novel object recognition test to measure the cognitive defect. All mice were euthanized at 4 months of age for analysis of biochemical and histological corrections. AAV vector biodistribution (copy numbers) was quantified by real-time PCR. **Results:** In the AAV-treated mice, GAA activity was extremely high in the heart (>26 folds of WT value) and restored to WT levels in the brain (cerebellum and cerebrum cortex), skeletal muscles, and liver (**Table 1**). High AAV copy numbers were detected in the brain, heart, and liver, but not in the skeletal muscles (**Table 1**). Consistent with these results, AAV treatment significantly reduced glycogen contents in these tissues: by >90% in the brain, nearly 100% in the heart, and 73-80% in skeletal muscles (**Table 1**). Periodic acid-Schiff (PAS) staining of brain sections from untreated GAA-KO mice reveals widespread lysosomal glycogen accumulations in the entire brain. Particularly, white matter and the Purkinje cell layer in the cerebellum, the glomerular layer of the olfactory bulb, and corpus callosum area show extensive glycogen accumulation. PAS-positive glycogen is mostly observed in the glial cells rather than neuronal cells in most brain regions, except the hindbrain (Pons and Medulla) where glycogen accumulates in both neuronal and glial cells. In the AAV-treated mice, no visible glycogen can be found in any regions of the brain. All the functional tests performed in this study showed significant improvement by the AAV treatment. **Summary:** For the first time, we demonstrated that a single intravenous injection of AAV-PHP.B vector into GAA-KO mice at a young age corrected disease phenotypes in both the brain and muscles. The AAV-PHP.B vector should also be effective for other neuromuscular and neurodegenerative disorders.

Table 1. Biochemical correction of glycogen storage in GAA-KO mice by AAV-PHP.B vector

	CB	CTX	Heart	Quad	Gast	Liver
UT [a]	2.79±0.16	3.78±0.22	10.73±4.07	5.78±0.82	5.25±0.58	4.99±0.37
AAV [a]	37.12±23.05	38.52±14.14	638.30±184.90	24.21±11.44	16.17±4.07	58.86±27.36
WT [a]	25.27±7.68	34.25±3.83	23.98±2.73	11.79±0.37	14.37±2.68	47.00±7.73
UT [b]	0.11±0.01	0.15±0.02	3.26±1.44	0.96±0.18	0.92±0.13	0.75±0.11[§]
AAV [b]	0.01±0.00	0.01±0.01	0.00±0.01	0.19±0.08	0.25±0.11	0.44±0.11[§]
AAV [c]	-	4.85±2.45	6.43±0.76	0.04±0.01	0.05±0.02	3.14±0.61

[a] GAA activity (nmol/h/mg protein); [b] Glycogen content (μmol glucose/mg protein); [c] AAV copy number per genome; [§] Non-fasting liver glycogen. Data represent as mean ± SD. Abbreviations: CB, cerebellum; CTX, cerebrum cortex; Quad, quadriceps; Gast, gastrocnemius; UT, untreated GAA-KO mice; AAV, AAV-treated GAA-KO mice; WT, wild-type mice.

839. Next-Generation Antibody-Guided Enzyme Replacement Therapy for Lysosomal Storage Diseases

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Pompe disease is a lysosomal disease caused by lysosomal glycogen accumulation due to acid alpha-glucosidase (hGAA) deficiency. Glycogen accumulation leads to progressive myopathy and damage to skeletal muscle, cardiac muscle, and some neurons. Enzyme replacement therapy (ERT) with recombinant human GAA protein is the primary treatment for Pompe patients. However, two major issues hinder the efficacy of hGAA ERT. First, hGAA is poorly delivered to skeletal muscle because the receptor mediating uptake of hGAA, the cation-independent mannose 6-phosphate receptor (CI-MPR), is poorly expressed in skeletal muscle. Second, some patients develop anti-rhGAA antibodies that can hinder uptake and cause immune reactions. Recently, liver depot gene therapy, where hepatocytes are transduced to produce soluble hGAA, has been shown to immune-tolerize Pompe mice to hGAA and can mitigate the formation of anti-hGAA antibodies. However, this technology was unable to fully correct skeletal muscle pathologies due to the inherent limitations of CI-MPR-mediated delivery, resulting in insufficient glycogen clearance. Here, we present an antibody-guided enzyme replacement therapy where antibodies are fused to hGAA and guide hGAA to skeletal muscle by targeting cell-surface internalizers that have a more favorable biodistribution and internalization kinetics than CI-MPR. Antibodies against broadly-expressed or skeletal muscle-specific internalizers were fused to hGAA and were able re-direct hGAA independently of CI-MPR *in vitro.* AAV-mediated liver depot gene therapy using an antibody::hGAA against the broadly expressed tetraspanin CD63 cleared glycogen in cardiac and skeletal muscles in Pompe mice to wild-type levels, while hGAA alone was only able to reduce 50% of muscle glycogen at the same dose. Markers for autophagy and lysosomal over-proliferation were significantly improved 3 months after the antibody::hGAA treatment. Antibody::hGAA treated mice show improved performance on Rotarod and grip strength within 2 months of treatment, performing similarly to wild-type mice, while hGAA treated mice only stabilized or declined their strength. This technology is generalizable to other lysosomal storage diseases and also amenable to depot-based cross-correctional gene therapy models.

840. *In Vivo* CRISPR-Based Dissection of a Metabolic Pathway

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Introduction: CRISPR-Cas9 is a flexible tool allowing the targeting of several loci for gene invalidation, modification or correction.

This advantage can be harnessed to further elucidate the function of metabolic pathways implicated in rare metabolic diseases. Deficiencies in enzymes along the canonical tyrosine degradation pathway lead to diseases, including alkaptonuria, hereditary tyrosinemia type I, and hereditary tyrosinemia type III. **Methods**: We designed a single vector strategy aimed at dissecting the tyrosine degradation pathway in a liver-specific manner. Systemic injection of recombinant AAV8 vectors expressing *Staphylococcus aureus (Sa)* Cas9 under the control of a liver-specific promoter was used to target 4-hydroxyphenylpyruvate dioxygenase (*Hpd)*, homogentisate 1,2-dioxygenase (*Hgd)* and glutathione transferase zeta 1 (*Gstz1)* in neonatal tyrosinemic *Fah*$^{-/-}$ mice by injection into the retro-orbital sinus. We determined the impact of such metabolic rewirering by measuring succinylacetone, homogentisic acid, tyrosine and phenylalanine levels, key catabolites of the pathway, using GC/MS. Liver and kidney morphology was also assessed following the removal of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), a potent inhibitor of HPD used to prevent lethality in tyrosinemic *Fah*$^{-/-}$ mice. **Results:** Stepwise metabolic rewirering of the tyrosine degradation pathway by invalidation of *Gstz1* or *Hgd* in *Fah*$^{-/-}$ mice exacerbated the phenotype and led to rapid death (6 and 3 days, respectively) following NTBC removal. Specifically, targeting *Gstz1* led to a massive increase in succinylacetone in *Fah*$^{-/-}$ mice treated while high levels of homogentisic acid were observed in *Hgd*-targeted mice. Animals in both groups also suffered from acute and lethal kidney failure following NTBC removal highlighting the systemic impact caused by the liver-based treatment. Metabolic rewirering of the pathway by invalidating *Hpd* rescued the phenotype of the *Fah*$^{-/-}$ mice as assessed by normal survival off NTBC, low levels of succinylacetone and proper liver and kidney function. **Conclusions:** SaCas9 is highly efficient *in vivo* and can be used to probe the function of metabolic pathways. This approach could be particularly useful for large-scale screen of hepatic therapeutic targets, by-passing the production of mouse models as well as the concerns of compensation related to embryonic transgenic models.

841. Phenotypic Correction by AAV8 Gene Therapy of a Mouse Model of Wilson Disease

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Wilson disease is an autosomal recessive disorder of copper transportation, which results in the accumulation of copper in the liver and other tissues due to mutations in the Wilson disease protein, a copper-transporting P-type ATPase (Atp7b). Current interventions treat Wilson disease by reduction of copper absorption or removal of excess copper from the body, but the underlying pathological process remains uncorrected and liver transplantation is required. For evaluation of the Wilson disease phenotype in a mouse model, we selected the toxic milk mouse (*tx*^{j}) available from Jackson Laboratories. These mice (called Atp7b KO) have a Gly712Asp missense mutation in the *Atp7b* gene, which is located in the second putative membrane-spanning domain of the encoded protein and results in a dysfunctional Atp7b protein. All Atp7b KOs were fostered from birth to allow for accurate determination of the time line of disease progression. Atp7b KO mice accumulate copper in the liver from birth, with severe copper accumulation seen by Timm's copper stain at two months of age. Liver disease then develops with release of copper into the serum, resulting in an apparent decrease in accumulation in hepatocytes and rising serum copper levels by 3-4 months of age. Hepatocellular hypertrophy, degeneration, and necrosis peak at 6 months of age, with the likely concomitant observation of areas of hepatic nodular regeneration from this age onwards. While there are marked increases in serum transaminases by 3-4 months of age, increases in serum total bilirubin levels only start to occur at 9 months, indicating advanced liver disease. A prophylactic gene therapy approach was attempted, but due to the large size of the *ATP7B* transgene, the length of the expression control elements had to be reduced to allow expression from an adeno-associated viral (AAV) vector. Atp7b KO mice were injected intravenously with AAV8 vector expressing a codon-optimized version of human *ATP7B* from a reduced size transthyretin promoter. Two-month-old mice were dosed at 10^9, 10^{10}, and 10^{11} genome copies (GC) per mouse and followed for 8 months post-vector administration. Following the conclusion of the study, mice were necropsied and liver samples were collected for histopathology. The higher doses of vector evaluated here (>10^9 GC/mouse) resulted in significant decreases in liver copper levels compared to age-matched, un-injected Atp7b KO mice. There was a significant, dose-dependent decrease in liver disease, with only mild histopathological findings present in male mice injected with 10^{11} GC/mouse of AAV8 vector with a complete lack of liver fibrosis. Therefore, administration of a gene therapy approach during the early stages of disease onset prevented liver damage and corrected copper metabolism in a mouse model of Wilson disease.

842. CRISPR/Cas9-Mediated Disruption of Glycolate Oxidase is an Efficacious and Safe Treatment for Primary Hyperoxaluria Type I

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Primary hyperoxaluria type I (PH1) is an inborn error of glyoxylate metabolism caused by mutations in *AGXT* gene that lead to the deficiency of hepatic alanine-glyoxylate aminotransferase (AGT) enzyme. In consequence, oxalate is overproduced in the liver and excreted in urine, which is deposited as calcium oxalate crystals in renal parenchyma, forms stones and progressively damages the kidneys until end-stage renal disease is developed. The only curative treatment

is liver transplantation. Substrate reduction therapy (SRT) has been explored as alternative treatment: the inhibition of glycolate oxidase (GO) enzyme, which produces glyoxylate (the precursor of oxalate) from glycolate, with siRNAs has shown beneficial therapeutic effect in the PH1 mouse model. However, this inhibition is transient. In this work, the newly discovered CRISPR/Cas9 systems have been applied to inhibit *Hao1* (the gene that codes GO) permanently at genomic level. For this purpose, AAV8 vectors carrying *Staphylococcus aureus* Cas9 and *Hao1*-specific guide RNAs have been generated and inoculated to PH1 animals. Treated animals presented an efficient edition of the *Hao1* locus, measured as the introduction of insertions and deletions (indels) in the coding sequence, which led to a dramatic decrease of GO protein levels. Moreover, as observed in previous studies, the inhibition of GO prevented oxalate overproduction and protected the mice against the accumulation of CaOx crystals during a challenge using an oxalate precursor. These effects were maintained up to 4 months after the treatment. NGS revealed the characteristics of the errors introduced in the on-target region and no off-target effect in *in silico* predicted top seven off-target regions. In conclusion, we have developed a safe and efficient CRISPR/Cas9-based SRT for PH1.

843. AAV-Directed Liver Gene Therapy for Crigler-Najjar Syndrome

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Crigler-Najjar (CN) syndrome is an extremely rare genetic liver disorder characterized by severe and life-threatening hyperbilirubinemia. Neurotoxic bilirubin accumulation is caused by a deficiency in the liver enzyme uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1), which is responsible for the conjugation and excretion of bilirubin. To date, there is no treatment for patients suffering from CN syndrome except for intensive phototherapy (up to 12 hours per day). Liver transplantation remains the only curative treatment. Here, we developed and optimized an adeno-associated virus (AAV) vector expressing UGT1A1 for the treatment of the CN. Safety and efficacy of liver gene transfer with AAV8-UGT1A1 were evaluated in preclinical animal models of CN and clinical trial-enabling studies initiated. In both UGT1A1 -/- mice and Gunn rats, results obtained demonstrate a safe and efficient gene therapy with durable correction of hyperbilirubinemia after one single administration of AAV-hUGT1A1 vector at the clinically relevant dose. Large-scale high-quality vector preparations were produced in HEK293 cells grown in suspension to support the GLP toxicology and biodistribution studies and other non-clinical studies including immunogenicity assessment and germline transmission evaluation. GMP lots of vector for clinical use were also produced and released. In parallel, ~50 patients were screened for anti-AAV8 neutralizing antibodies to evaluate the number of potentially eligible subjects. These studies allowed for the initiation of a phase I/II trial in severe CN subjects.

844. A Patient-Tailored Mouse Model to Enable Development of Gene and Cell Therapies for Barth Syndrome

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Barth Syndrome (BTHS) is an X-linked pediatric disorder characterized by growth delay, muscle weakness, neutropenia, and cardiomyopathy, and is associated with a high risk of mortality in early life due to infection or heart failure. BTHS is caused by inherited mutations of *tafazzin* (*TAZ*), which encodes a phospholipid-lysophospholipid transacylase required for remodeling of the mitochondrial phospholipid cardiolipin. No specific treatments for BTHS exist, and medical management relies on prophylactic prevention of infections and symptomatic treatment of cardiomyopathy. Development of therapies for BTHS has been impeded by lack of a genetic animal model. We therefore utilized CRISPR/Cas9 technology to introduce a BTHS patient-specific point mutation into the endogenous murine *Taz* gene, generating mice that bear a pathogenic amino acid substitution (D>H) within the conserved D75 residue of Taz. We have previously reported that Taz^{D75H} male mice recapitulate the chief manifestations of BTHS, including neutropenia and impaired cardiac function in early life. Here we further refine the characterization of this model. Our previous work found prenatal cardiac defects and cardiomyopathy in young Taz^{D75H} males. Here we find that cardiac function further deteriorates in aged Taz^{D75H} males, with echocardiograms demonstrating more severely reduced fractional shortening ($p=0.0197$) and ejection fraction ($p=0.0233$). Impaired granulopoiesis of young Taz^{D75H} males was validated via transplantation of Taz^{D75H} into wild-type recipients: Taz^{D75H} recipients exhibited significantly reduced absolute neutrophil counts relative to control transplanted mice one month post transplant ($p=0.0104$). Hematopoietic dysfunction of Taz^{D75H} mitochondria was confirmed by extracellular flux analysis of bone marrow-derived macrophages (BMDMs), which revealed a significant reduction in the basal oxygen consumption rate of Taz^{D75H} BMDMs relative to wild-type ($p<0.0001$). Transmission electron microscopy visualized dysmorphic mitochondria in Taz^{D75H} skeletal muscle and hearts. Morphometric analysis of Taz^{D75H} skeletal muscle mitochondria revealed an increase in cristae width ($p<0.0001$), and mitochondria from Taz^{D75H} male hearts exhibited vesicular cristae that formed a striking honeycomb pattern (**Fig. 1**; $p<0.0001$). Mitochondrial defects characteristic of BTHS are likely traced to impaired cardiolipin metabolism. Liquid chromatography-mass spectrometry analysis revealed elevated levels of immature cardiolipin relative to mature cardiolipin in the peripheral blood of male Taz^{D75H} mice ($p=0.0004$), demonstrating a severe impairment of cardiolipin metabolism, a key criterion of

BTHS diagnosis. Further evidence of metabolic derangement in BTHS patients includes a high incidence of 3-methylglutaconic (3-MGA) aciduria. Likewise, we found increased levels of 3-MGA in urine of Taz^{D75H} mice (p=0.0089). Untargeted primary metabolism analysis revealed an accumulation of citric acid cycle intermediates in the plasma of Taz^{D75H} mice, consistent with impaired mitochondrial respiration. Together, these findings establish that Taz^{D75H} mice fully recapitulate the multisystemic spectrum of BTHS symptoms and will serve as a platform to elucidate the mechanisms of disease pathogenesis and to develop gene and cell therapies specific to *Taz* deficiency.

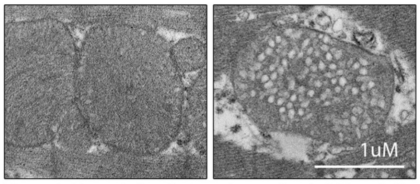

Figure 1. Transmission electron micrographs of wild-type (left) and Taz^{D75H} (right) cardiac cross sections.

845. AAV9 Intracerebroventricular Gene Therapy Improves Lifespan and Normalises Long-Term Locomotor Behaviour in a Mouse Model of Niemann-Pick Type C1 Disease

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Niemann-Pick Type C (NP-C) is a fatal lysosomal storage disorder with neurovisceral pathology, for which there is currently no major disease modifying treatment. In the majority of cases, loss of the late endosomal transmembrane NPC1 protein causes systemic intracellular lipid accumulation. Premature death is normally associated with progressive neurodegeneration and subsequent neurological disease manifestations. This project focuses on the development and pre-clinical evaluation of gene delivery to the brain in a well-characterised mouse model of NP-C using an adeno-associated viral (AAV) vector. A single administration of an AAV9 vector expressing the human NPC1 cDNA via a neuron-selective promoter into the brains of neonatal wild-type mice resulted in successful over-expression of the human NPC1 protein. Supraphysiological expression of exogenous NPC1 was shown to not cause adverse effects in administered brains and produced NPC1 correctly localised to within late endosomal/lysosomal compartments. Administration in neonatal *Npc1*$^{-/-}$ mice resulted in a significant extension of average lifespan by over 120%, compared to untreated *Npc1*$^{-/-}$ mice ($p < 0.0001$). AAV-mediated gene therapy also prevented or ameliorated neurodegeneration in all monitored brain regions, resulting in the normalisation of various indices of motor function throughout their lifespan, along with significant improvements in weight and biochemical pathology. Additionally, the therapeutic efficacy of this gene therapy study was directly compared to competing NP-C therapies. These proof of concept results demonstrate the use of gene therapy as a potential therapeutic option for clinical use in Niemann-Pick type C and support the further development of this approach.

846. AAV-Mediated Liver Expression of Secretable GAA Shows Therapeutic Efficacy in Pompe Mice at Low Vector Doses

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Pompe disease is a neuromuscular and lysosomal storage disease caused by mutations in the gene encoding for acid α-glucosidase (GAA). GAA breaks down lysosomal glycogen into glucose, and its deficiency leads to glycogen buildup all over the body. Newborn infants who are affected by Pompe disease present with severe cardiomegaly, muscle weakness, respiratory impairments and neurological defects which lead to a premature death. Conversely, late-onset of Pompe disease (LOPD) patients show progressive muscle and respiratory impairments with no cardiac manifestations. Enzyme replacement therapy (ERT) is a life-saving treatment for patients with infantile-onset of Pompe disease and stabilizes the disease phenotype in LOPD patients, however it suffers from several shortcomings, including limited efficacy and high immunogenicity. We recently reported correction of Pompe disease in symptomatic four month-old $Gaa^{-/-}$ mice by adeno-associated virus (AAV) vector liver gene transfer of secretable GAA. Whole-body therapeutic efficacy was achieved at the dose of $2x10^{12}$ vg/kg upon long-term treatment (*Puzzo, Colella et al., Sci Trans Med, 2017*). In order to further investigate the therapeutic potential of our liver gene therapy approach with secretable GAA, we treated four month-old mice with low vector doses ($1x10^{11}$ and $5x10^{11}$ vg/kg) of AAV expressing either secretable or native GAA transgenes. Six months after treatment, the survival of $Gaa^{-/-}$ mice was preserved only by treatment with AAV vectors encoding for secretable GAA, at both vector doses. Conversely, $Gaa^{-/-}$ mice treated with a vector encoding for native non-secretable form of GAA displayed a mortality rate comparable to the PBS-treated $Gaa^{-/-}$ mice (50% at 6 months after treatment; p-value<0.05). Survival was not accompanied by significant improvement of muscle strength in $Gaa^{-/-}$ mice treated with the lowest dose of AAV-GAA ($1x10^{11}$ vg/kg). Complete rescue of muscle strength was instead observed in $Gaa^{-/-}$ mice treated with $5x10^{11}$ vg/kg of AAV expressing secretable GAA. Conversely, muscle strength was not improved in $Gaa^{-/-}$ mice treated with AAV encoding for native non-secretable GAA at a vector dose of $5x10^{11}$ vg/kg. These results demonstrate a clear dose-response of AAV-GAA liver gene transfer in the Pompe mouse model and highlight the superior therapeutic efficacy of secretable GAA compared to native non-secretable GAA.

847. Split AAV-Mediated Gene Addition Therapy for Carbamoyl Phosphate Synthetase 1 Deficiency

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The liver is one of the most important organs of the body, regulating dozens of aspects of metabolism throughout development and adulthood, including the urea cycle, the major pathway by which waste nitrogen is eliminated. Loss or dysfunction of enzymatic activity in this pathway, known as urea cycle disorders (UCDs), leads to elevated ammonia, imbalanced amino acids, cerebral edema and neurological decline, ataxia, and eventually death if untreated. Carbamoyl phosphate synthetase 1 (CPS1) catalyzes the first committed step of the urea cycle, and its disruption typically leads to the most severe symptoms of the UCDs. Current medical management is extremely challenging with the only effective long-term treatment strategy being orthotopic liver transplantation; however, donor availability and the potential for short- and long-term complications severely limit its efficacy. To address the need for novel therapeutic options for CPS1 deficiency, we are using an adeno-associated virus (AAV) approach to deliver functional human codon optimized CPS1 cDNA (hcoCPS1) into deficient hepatocytes. We chose AAV to circumvent the issues of low titer and random genomic integration by lentiviruses, as well as immunogenicity of helper-dependent adenoviruses. The CPS1 cDNA is relatively large (4.5 kb), and previous attempts by our group to develop a functional AAV as a vector of 5.1 kb failed to produce CPS1 protein. To overcome the inherent size limitation of AAV packaging, we will use a split AAV (sAAV) approach that capitalizes on the natural recombinogenic potential of AAVs (Figure 1). The full-length promoter, cDNA, and polyA will be split into 2 halves, each being packaged separately into individual viruses. When cells are co-transduced by both viruses, the viruses concatemerize to reconstitute hcoCPS1. sAAVs may be designed to recombine by either trans-splicing or overlapping. Trans-splicing flanks the ITRs with intronic sequences that results in their removal from mature mRNA (Figure 1A). Overlapping causes homologous recombination of the two viruses in the nucleus and the subsequent loss of the ITRs. We are testing each approach to determine which will lead to higher hcoCPS1 expression in transduced cell lines *in vitro* and measuring gene and protein expression. The approach with the higher expression will be used *in vivo* to transduce conditionally CPS1 deficient mice to determine if provision of hcoCPS1 using sAAV is sufficient to ameliorate symptoms of CPS1 deficiency and restore ureagenesis in a mouse model of the disease.

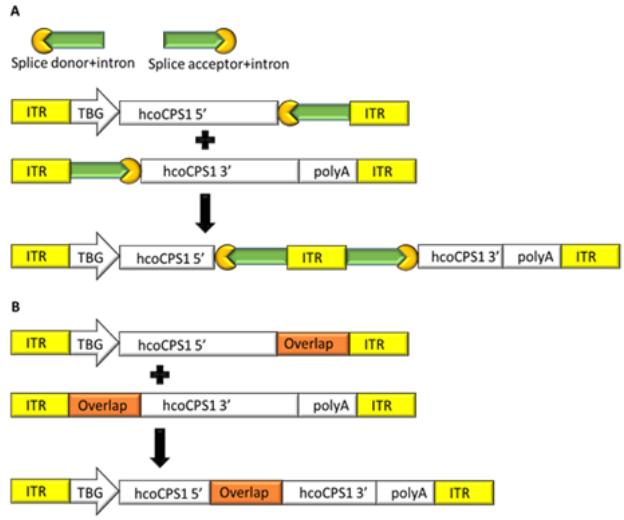

Figure 1. sAAV design. A) sAAV trans-splicing approach which removes ITRs by splicing them out. B) sAAV overlapping approach which removes ITRs by homologous recombination within the transgene. ITR, inverted terminal repeat; TBG, thyroxin binding globulin; hcoCPS1, human codon optimized CPS1

848. Cognitive and Motor Assessment in a Novel Murine Model of GM1 Gangliosidosis Developed Utilizing CRISPR-Cas9 Genome Editing

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GM1 gangliosidosis (GM1) is a lysosomal disease caused by mutations in the GLB1 gene, which encodes the lysosomal hydrolase β-galactosidase (β-gal; EC 3.2.1.23). Insufficient β-gal catalytic activity results in the accumulation of the gangliosides GM1 and GA1 within the nervous system, resulting in progressive neurodegeneration and death. Treatments for this debilitating disease are not currently available, thus the development and testing of novel therapies in a model organism is of grave importance. To generate a mouse model of GM1, CRISPR/Cas9 genome editing was used to target exon 8 of the *Glb1* gene. Of the 106 zygotes that were injected, one animal harbored a 20 bp frame-shift deletion that encompassed the predicted catalytic residue of β-gal. Subsequent analysis of enzyme activity in β-gal deficient animals (β-gal-/-) showed that this mutation resulted in nearly a complete loss of β-gal enzyme activity (0-1% of wildtype levels, and 0-2% of heterozygous). Over the course of 6 months, animals were monitored for weight and behavioral changes, at which time they were tested with a battery of neurocognitive and motor function tests to elucidate the severity and phenotype of the disease. By 6 months of age, animals displayed features of a neurological disease, such as ataxia, bodily tremors, and abnormal gait. Neurocognitive testing using the Barnes maze and spontaneous alternation in the T-maze showed that β-gal(-/-) mice had significant spatial reference memory and spatial working memory impairments. To test motor function, animals were subjected to four tests: the balance beam, pole, inverted screen, and rotarod; which showed that β-gal mice have significant motor

impairments. Overall, the results of this study show that the β-gal(-/-) mice harboring the 20 bp deletion recapitulate many phenotypes of human GM1, making it a compelling model for potential future gene therapy studies.

849. Nonclinical Safety Evaluation of VTX-801, an AAV Vector for Treatment of Wilson's Disease

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Copper is an essential trace element to the proper functioning of organs and metabolic processes. All organisms and cells have complex homeostatic mechanisms to ensure a constant concentration of available copper, while eliminating excess copper whenever this occurs. Like all essential elements, too much or too little copper can result in corresponding conditions of copper excess or deficiency, each of which has its own unique set of adverse health effects. A recent report described lowered hepatic copper concentrations in patients with non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) (Aigner et al., 2010). The authors showed that lower hepatic copper levels in patients were associated with more pronounced hepatic steatosis, NASH, and components of the metabolic syndrome. They also reported development of hepatic steatosis and insulin resistance in response to dietary copper restriction in rats, suggesting that impaired copper availability may be involved in the development of NAFLD. Furthermore, low copper levels leads to imbalances in iron homeostasis and anemia (Matak et al. 2013). Wilson's disease (WD) is a copper metabolic inherited disease due to mutations in the copper transporter ATP7B. We have recently developed an AAV-Anc80 vector carrying a smaller version of the protein, named VTX-801, which provides long-term correction of copper metabolism in a WD mouse model. Here, we evaluated the potential toxicity of WD gene therapy vector VTX-801 in male and female C57BL/6 mice treated with three different doses of VTX-801. The therapeutic effect was evaluated for three months by testing blood samples for ceruloplasmin activity, hematology, clinical chemistry, with a specific focus on liver function. At time of sacrifice, copper concentration in the liver and hepatic histology were also analyzed. All the different serum parameters analyzed were normal, i.e. no significant differences with untreated animals were observed. Furthermore, copper hepatic content was similar in all groups independently of the treatment. Liver histology was completely normal, even in animals treated with the higher dose of VTX-801. No signs of hepatic steatosis, liver inflammation or cell death were observed. In summary, overexpression of a mini ATP7B transporter did not alter copper metabolism, indicating that even in the presence of an excess of transgene, hepatocytes have mechanisms in place to ensure a proper copper homeostasis. This provides further support for VTX801 as a candidate for the treatment of WD.

850. Engineered Epidermal Progenitor Cells Can Correct Diet-Induced Obesity and Diabetes

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Somatic gene therapy is a promising approach for treating otherwise terminal or debilitating diseases. The human skin is a promising conduit for genetic engineering, as it is the largest and most accessible organ, epidermal autografts and tissue-engineered skin equivalents have been successfully deployed in clinical applications, and skin epidermal stem/progenitor cells for generating such grafts are easy to obtain and expand in vitro. Here, we develop skin grafts from mouse and human epidermal progenitors that were engineered by CRISPR-mediated genome editing to controllably release GLP-1 (glucagon-like peptide 1), a critical incretin that regulates blood glucose homeostasis. GLP-1 induction from engineered mouse cells grafted onto immunocompetent hosts increased insulin secretion and reversed high-fat-diet-induced weight gain and insulin resistance. Taken together, these results highlight the clinical potential of developing long-lasting, safe, and versatile gene therapy approaches based on engineering epidermal progenitor cells.

Musculo-Skeletal Diseases II

851. Implications of Dystrophin Hinge Regions in Micro-Dystrophin Gene Replacement Therapy for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is an X-linked muscle disease resulting from the loss of cytoskeletal protein dystrophin. The dystrophin gene is one of the largest genes in the human genome. Dystrophin protein consists of four regions; amino terminus (NT), central rod domain with 24 spectrin-like repeat (R) regions and four hinge (H) regions, cysteine rich domain (CR) and carboxyl- terminal domain. While the biological functions of NT, CR and CT as well as repeats in the rod domain and H2 and H3 have been extensively interrogated, little is known about the function of H1 and H4. Using adeno-associated virus (AAV) delivery as a platform, we studied the structure-function relationship of H1 and H4 in the context of micro-dystrophin. We started with a microgene carrying both H1 and H4. A series of new microgenes with full or partial H1 and/or H4 deletion were generated. All microgenes were delivered to the tibialis anterior muscle of 3-m-old male mdx4cv mice using AAV-9 at the dose of 1E12 viral genome particles per muscle. Muscle force was evaluated at 6-month post injection. Compared with the parental construct, complete deletion of H1 did not alter micro-dystrophin function, whereas complete deletion of H4 compromised force rescue by micro-dystrophin. Some partial deletion constructs yielded better muscle force rescue while others showed similar rescue as that of

the parental construct. These data suggest that H1 and H4 play an important role in muscle function (Supported by NIH and Jackson Freel DMD Research Fund).

852. Restoration of Functional Glycosylation of Alpha-Dystroglycan in FKRP-Deficient iPSCs Using CRISPR/Cas9

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Background: Fukutin-related protein (FKRP) is a ribitol-5-phosphate (Rbo5P) transferase that plays a key role in the glycosylation of α-dystroglycan (α-DG), a cell surface glycoprotein that stabilizes the interactions of muscle and CNS cells to laminin in the surrounding extracellular matrix (ECM). FKRP mutations that disrupt glycosylation of α-DG impair adhesion of neurons and skeletal muscle to the ECM. Depending on the severity of α-DG glycosylation impairment, FKRP mutations manifest in dystroglycanopathies ranging from milder forms such as limb-girdle muscular dystrophy 2i (LGMD2i), which is characterized by progressive muscle weakness and atrophy during early adolescence, to more severe diseases such as Walker-Warburg Syndrome (WWS), which is associated with severe developmental defects in the CNS and muscular system. Currently, there are no available therapies for these patients. **Strategy**: As FKRP-based dystroglycanopathies are autosomal recessive disorders, correction of a single gene copy is expected to restore cell function. Our strategy involves CRISPR/Cas9 mediated knock-in of a repair cassette into intron 3 of the FKRP gene. The repair cassette contains the WT FKRP coding sequence flanked by a β-globin splice acceptor sequence and a polyadenylation signal. Based on the splice capture approaches followed in previous gene therapy studies, we anticipate that transcription from the endogenous promoter will preferentially incorporate the exon from the repair cassette instead of the mutant FKRP exon into the spliced mRNA, thereby restoring gene function. Since FKRP mutations are distributed throughout the coding region, our strategy should be effective in many patients. Our approach will be advantageous over episomal expression from AAV vectors and targeted correction of the mutation in the FKRP exons for three reasons. First, correction at the genomic locus will result in a permanent restoration of FKRP function, in contrast to the potentially transient expression from AAV vectors in proliferating muscle stem cells, like satellite cells. Second, correction of FKRP mutations at the endogenous locus would result in expression of FKRP at physiological levels. Third, introducing the repair cassette in the intronic region would avoid potential damage to the FKRP locus for uncorrected alleles. **Results**: As a first step toward defining an effective strategy for correcting FKRP mutations in a mouse model, we tested our gene correction strategy on WWS iPSCs. These FKRP null cells are severely deficient in α-DG glycosylation, permitting quantitative assessment of FKRP functional rescue. We used CRISPR/Cas9 guide ribonucleoprotein (RNP) complexes and ssDNA donor templates to insert the FKRP gene correction cassette into intron 3 of FKRP gene. Successful knock-in of the correction cassette and expression of the corrected FKRP mRNA was detected by genotyping and qRT-PCR respectively. We also observed successful restoration of α-DG glycosylation in the corrected cell population using flow cytometry assays. **Conclusions:** We have demonstrated a general approach for the functional correction of FKRP mutations in WWS iPSCs using CRISPR/Cas9 and a donor template that should be agnostic to the type of inactivating mutation within the coding sequence. These experiments establish a foundation for testing this FKRP-correction strategy *in vivo* using a mouse model. These pre-clinical studies will ultimately inform the application of FKRP gene repair technology for the treatment of FKRP-based dystroglycanopathies.

853. 3D Printed Tricalcium Phosphate Scaffolds and Regional Gene Therapy. a Multidisciplinary Approach to Heal a Critical-Sized Rat Femoral Defect Model

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Introduction: This study evaluated the efficacy of a three-dimensionally (3D) printed tricalcium phosphate (TCP) scaffold loaded with rat bone-marrow cells (RBMCs) genetically modified to overexpress BMP-2 to promote bone repair. Methods: TCP scaffolds were 3D printed with 500 micron pores and dimensions to specifically fit a critical sized rat femoral defect. RBMCs were transduced with a lentiviral vector expressing BMP-2 (LV-BMP-2) or GFP (LV-GFP). A critical-sized femoral defect was created in Lewis rats, randomized into four treatment groups: TCP+RBMC/LV-BMP-2 (Group I, n=14), TCP+RBMC/LV-GFP (Group II, n=5), TCP+non-transduced RBMCs (Group III, n=5), and TCP scaffold alone (Group IV, n=5). The femora were evaluated at twelve weeks with use of radiography, microcomputed tomography (microCT), histology, histomorphometric analysis, and biomechanical testing. Results: All TCP+LV-BMP-2 samples demonstrated complete union of the femoral defect on radiography and microCT (Figures 1 and 2). No femora healed in groups II-IV (Figures 1 and 2). MicroCT revealed that TCP+LV-BMP-2-treated animals formed 197% greater bone volume when compared to groups II-IV ($P<0.05$). Histology demonstrated cortical bridging of the scaffold to the intact femur proximally and distally in the TCP+LV-BMP-2 samples. Histomorphometric analysis confirmed the presence of higher bone volume in the TCP+LV-BMP-2 group when compared to groups II-IV ($P<0.05$). Biomechanical testing demonstrated equivalent stiffness of the healed femur treated with TCP+LV-BMP-2 when compared to the contralateral intact control femur ($P=0.863$). Torque and energy to failure, and peak displacement were greater in the contralateral intact control femur ($P<0.001$). Conclusions: A bioengineered, osteoconductive 3D printed TCP scaffold combined with a highly osteoinductive signal achieved through cell transduction can successfully heal a critical sized rat femoral defect. The ability to

3D print scaffolds contoured to fit specific anatomic defects combined with regional gene therapy could have significant clinical potential for bone regeneration.

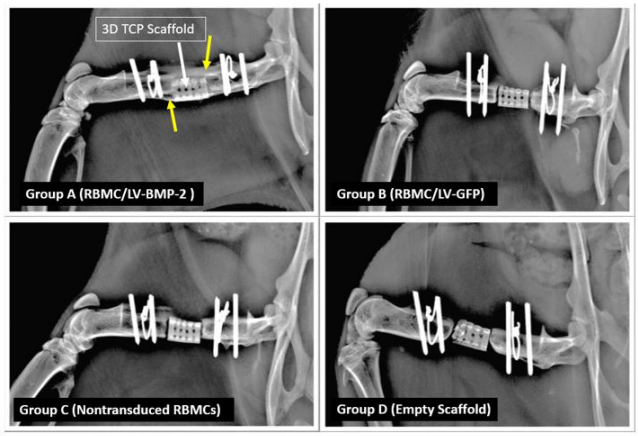

Figure 1. Group A demonstrates complete radiographic healing of the critical sized rat femoral defect at 12 weeks. There is cortical bone (yellow arrows) surrounding the 3D printed scaffold (white arrow). There is no evidence of radiographic healing in groups B-D.

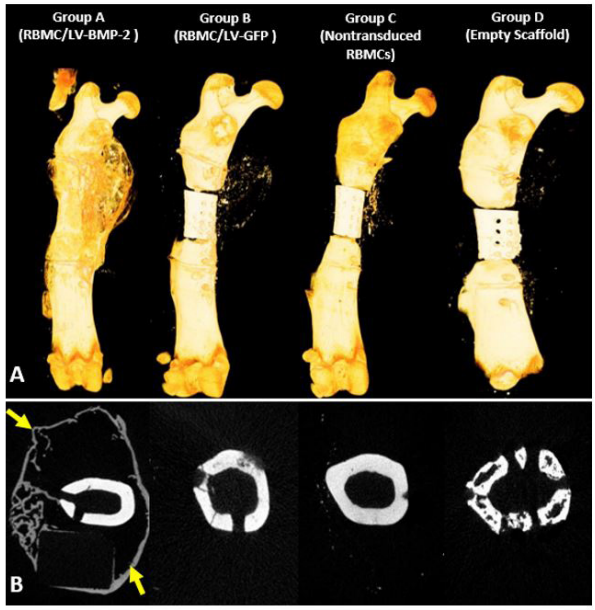

Figure 2. A.) MicroCT 3D reconstruction images confirming complete healing of the critical sized rat femoral defect at 12 weeks in Group A. There is no bone formation surrounding the 3D printed scaffold in Groups B-D. **B.)** MicroCT axial images of the scaffold at the midpoint of the rat femoral defect. In Group A there is circumferential bone formation (yellow arrows) around the 3D printed scaffold. There is no evidence of bone formation around the 3D printed scaffold in Groups B-D.

854. Preclinical Evaluation of SGT-001 Microdystrophin Gene Transfer for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a severe muscle disorder caused by loss-of-function mutations in the DMD gene, which lead to the absence of the dystrophin protein. Without dystrophin, the structural link between the actin cytoskeleton and extracellular matrix is broken, and muscles are highly susceptible to contraction-induced damage. Direct dystrophin replacement has thus far been unsuccessful due to the large size of the DMD gene. As a wide variety of mutations can be responsible for the disease, therapeutic strategies aimed at specific mutations only have the potential to benefit subsets of patients. SGT-001 is an adeno-associated virus (AAV) microdystrophin gene transfer candidate being evaluated by Solid Biosciences in a Phase I/II adaptive clinical trial, called IGNITE DMD. The program is based on extensive research on the dystrophin protein, to develop shorter yet functional microdystrophin variants, and to deliver genes to muscle. This represents a unique strategy to benefit DMD patients regardless of dystrophin mutation. Significant preclinical work has been conducted to characterize SGT-001. Solid has performed studies in dystrophic mouse and dog models to evaluate biodistribution and microdystrophin protein expression following systemic administration. Preclinical data show that a single dose of SGT-001 leads to widespread biodistribution and long-term microdystrophin expression in skeletal and cardiac muscle. In dystrophic dog studies carried out for over two years, data show that this response is sustained throughout the entirety of the study. Quantification of protein expression using immunofluorescence, Western blotting and mass spectrometry identified a dose-response increase in percent positive muscle and overall protein levels. In addition, in vivo and in vitro functional assessments have demonstrated improved muscle function in a similar dose-dependent manner. Solid is developing SGT-001 to potentially become a viable therapeutic option for DMD patients, regardless of mutation.

855. AAV-RNAi Vector Development and Therapeutic Target Evaluation for Myotonic Dystrophy

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Myotonic dystrophy type I (DM1) is caused by a microsatellite expansion in the *dystrophia myotonica protein kinase (DMPK) gene*. Dominant disease results from 'gain of function' toxicity of the 3' UTR CUG repeat expansion *DMPK* mRNA. Repeat expanded mRNA (REmRNA) exerts its dominant effect by sequestering splicing factors, and limit their function, in what are referred to as nuclear inclusions or 'foci.' Recent efforts to develop a therapy for DM1 with an antisense oligonucleotide approach showed promise in preclinical studies in mice, but failed in phase I clinical trials. Alternatively, we have focused on optimizing an efficient delivery system using AAV6 coupled with engagement of the RNA interference pathway (RNAi). We previously showed that systemic delivery of AAV RNAi hairpin cassettes targeting the human *α-skeletal actin gene* (HSA) in the HSALR (long repeat) transgenic mouse model of DM can ameliorate myotonia and splicing alterations caused by REmRNA-related splicing factor inactivity. A new study demonstrates enhancement of REmRNA knockdown in the HSALR through blocking reporter gene expression which caused an unrelated RNAi-independent toxicity. In this study we compared our functional AAV-DM10 RNAi vector with and without reporter

gene expression by intramuscular injection of comparable doses in the tibialis anterior muscle of HSALR mice. Analysis of *Atp2α1* mRNA splicing demonstrated a 50% reduction of the aberrant neonatal splice product in the vector expressing the reporter gene compared to a 95% reduction without reporter gene expression at a dose of $5x10^{10}$ vector genomes. Based on results demonstrating increased efficacy of these vectors, we began developing RNAi hairpins targeting the human *DMPK* mRNA as the disease target for clinical development. Over 40 targets for RNAi were designed and are currently being evaluated for *DMPK* mRNA knockdown in HEK293 cells. Promising candidates will be further evaluated in DM patient immortalized myoblasts and myotubes for foci reduction and splicing reversion to adult patterns. Several improvements in vector design coupled with RNAi target development hold the potential to yield promising clinical candidates for AAV-RNAi DM1 gene therapy for delivery to the spectrum of tissues affected by *DMPK* RNA toxicity.

856. Effect of hISPD Gene Transfer on Glycosylation of α-Dystroglycans in a FKRP Dystroglycanopathy Mouse Model

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Dystroglycanopathy, a subgroup of muscular dystrophy, is characterized by a common biochemical feature, defective glycosylation of α-dystroglycans (α-DG), which reduces its binding activities to ligand proteins in the extracellular matrix, including laminins. This causes progressive loss of muscle integrity and functions. Currently, mutations in at least eighteen genes have been identified as responsible for dytroglycanophathy, including the genes encoding fukutin (FKTN) and fukutin-related protein (FKRP). FKTN and FKRP contribute to the synthesis of the glycan chain on the α-DG by transferring ribitol phosphate (ribitol-5P) from the donor substrate CDP-ribitol, which is synthesized by Isoprenoid synthase domain-containing protein (ISPD). Although mutations in FKRP leads to lack of functionally glycosylation α-DG (F-α-DG), the protein retains partial function to restore glycosylation of α-DG. We hypothesized that overexpression of hISPD in muscles in vivo, might increase the levels of CDP-ribitol substrate, enhancing the transfer rate of ribitol-5P to α-DG by mutant FKRP, thus compensating for its reduced functional activity. We tested this hypothesis in our FKRP P448L mutant mouse model, which is associated with severe congenital muscular dystrophy (CMD) in clinic. Our preliminary results show a detectable enhancement of α-DG glycosylation in cardiac muscles of mice systemically injected with AAV-hISPD after 3 months treatment. Our data indicates that the production of CDP-ribitol might be a limiting factor on the process of α-DG glycosylation and could be further explored as alternative strategy for experimental therapy to restore F-α-DG in dystroglycanopathies caused by FKRP mutations.

857. Exosomes in Human Urine Contain mRNA Splice Variant Biomarkers of Muscular Dystrophies

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Introduction: Extracellular vesicles (EVs) in biofluids contain mRNA and non-coding RNA that can serve as genetic biomarkers of glioblastoma, prostate cancer, and other disease states. In clinical trials for Duchenne muscular dystrophy (DD) and myotonic dystrophy type 1 (DM1), mRNA splicing outcomes in serial muscle biopsies are used to monitor therapeutic antisense oligonucleotide (ASO) drug effects. However, tissue biopsies are invasive, impractical for long term monitoring of therapeutic response, and require general anesthesia in children. We examined whether mRNA splice variants in EVs (ex-mRNA) of human serum or urine could meet sensitivity and specificity as robust markers of muscular dystrophies. **Methods**: We isolated ex-mRNA from serum and urine samples of individuals with DM1 (N = 34), Duchenne or Becker muscular dystrophy (DD/BD, N = 15), and unaffected (UA; N = 28) controls, examined gene expression by droplet digital PCR (ddPCR), and quantitated mRNA splicing outcomes by RT-PCR and ddPCR. Using a training set, principle component regression, and ROC analysis, we formed a composite biomarker and developed a predictive model of urine splicing outcomes in DM1. An independent validation set was used to test the model. We examined *DMD* deletion transcripts by ddPCR and sequencing of RT-PCR products. **Results**: In our training set, we identified 10 transcripts that are spliced differently in urine from DM1 patients as compared to DD, BD, or UA individuals. Our predictive model was 100% accurate in our independent validation set. By contrast, alternative splicing of all transcripts examined in serum was similar in DM1 and UA subjects. Urine also contains *DMD* deletion mRNAs that are mutation-specific and confirm exon-skipping activity of the antisense oligonucleotide eteplirsen. **Conclusions**: Urine provides a renewable source of ex-mRNA splice events that can serve as a powerful composite biomarker of DM1, suggesting its potential to monitor therapeutic response. Non-invasive measurements of splicing outcomes may expedite clinical trials to children with DM1, enable convenient titration of ASO dose during the course of treatment, and facilitate development of newer and better exon-skipping drugs for DD.

858. Allele-Specific *COL6A1* Gene Editing to Inactivate a Dominant Glycine Mutation Causing a Collagen VI-Related Muscular Dystrophy

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Collagen VI-related muscular dystrophies (COL6-RD) are congenital disorders of the muscle that result in significant weakness at birth or in early infancy, and for which there are currently no effective treatments. Recessive or dominant-negative mutations in any of

the main collagen 6 genes (COL6A1, COL6A2, COL6A3) underlie COL6-RD. As is the case with other collagens, glycine substitutions at specific positions on the collagenous domains of the COL6 genes are frequent, and exert a strong dominant-negative effect on collagen VI function. As a potential therapeutic approach, we used CRISPR/Cas9 to selectively introduce null edits on a mutant COL6 allele, with the aim of converting the dominant-negative state to an haploinsufficient one, which for the COL6 genes is clinically asymptomatic. We selected four patients carrying the common COL6A1 G290R (c.868G>A) mutation, and cultured primary skin fibroblasts from these patients. In muscle, collagen VI is mainly produced by the interstitial fibroblasts, thus dermal fibroblasts represent a valid model to study COL6 mutations. We designed two guide RNAs (gRNAs) specifically targeting the mutant coding sequence (c.868A), and cloned each gRNA into a SpCas9/GFP-expressing vector that we transfected into patient fibroblasts. We sorted for GFP+ cells, and then either extracted DNA for targeted re-sequencing (Illumina MiSeq platform), or cultured cells in serially diluted plates to isolate clonal populations. We subsequently analyzed clonal populations for collagen VI matrix production using immunofluorescence and immunoblotting. We found that deletions of a single nucleotide at the Cas9 cutting site (delC for gRNA-1, delG for gRNA-2) were the most frequently observed edits, and that these occurred on the mutant allele (c.868A) at a higher frequency, showing allele selectivity. We successfully isolated clonal cell populations in which the mutant allele was inactivated with a frameshifting edit. In these clones, the mutant allele expression was completely abolished, and the collagen VI matrix secretion phenotype was rescued in culture. Here, a single treatment with a gene editing enzyme introduced permanent edits, the majority of which led to inactivation of the mutant allele. We have previously described individuals mosaic for COL6 dominant-negative mutations, who displayed a significantly milder phenotype as opposed to patients heterozygous for the same mutations. As a potential treatment in vivo, inactivating the dominant-negative allele in even a fraction of cells could serve as a viable option to improve the clinical outcome, provided the high allele selectivity and the successful delivery of the gene editing components to muscle interstitial fibroblasts. This study further supports the hypothesis that suppressing a glycine mutation in the collagenous Gly-X-Y motif in COL6 blunts the dominant-negative effect on collagen VI function, which could be applied to other collagens in which haploinsufficiency leads to milder phenotypes.

859. Systemic AAV-Mediated Co-Delivery of Microdystrophin and CRISPR/Cas9 in a Mouse Model of DMD

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We and others have previously demonstrated that AAV-mediated, muscle-specific dystrophin gene editing using the CRISPR/Cas9 system can be utilized to induce dystrophin expression in mouse models of Duchenne muscular dystrophy (DMD). So far, therapeutically relevant dystrophin expression levels have mostly been demonstrated following direct intramuscular delivery of CRISPR/Cas9-encoding vectors, where extremely high-dose vector delivery is relatively easy to achieve. Systemic gene editing for treatment of all affected muscle groups is significantly more intricate due to several factors, including the delivery of very high vector doses to ensure optimal gene editing efficiency. While previous murine gene replacement studies using AAV vectors to deliver microdystrophins have effectively determined parameters needed for near complete transduction and transgene expression in all muscle groups, optimal conditions for AAV-mediated gene editing using the CRISPR/Cas9 system still need optimization due to unique aspects of this genome modifying system. First, delivered AAV vector(s) must express both Cas9 and guide RNAs to target genomic DNA and repair or bypass the disease causing mutation(s). Due to the limited packaging capacity of AAV, Cas9 and sgRNA expression cassettes often need to be separated into two different vectors for co-delivery, which increases the total vector dose needed for optimal transduction and hence demand for higher vector titers to reduce the injected volume. Additionally, CRISPR/Cas9 approaches are also limited by gene editing efficiency and the rate at which editing occurs after target cell transduction. Our observations suggest that while widespread dystrophin expression occurs in multiple muscle groups following systemic gene editing, expression levels in skeletal muscles appear significantly reduced compared to those achieved following direct intramuscular injection. These levels are further reduced over time, indicating a continuation of muscle turnover due to inadequate dystrophin-correction failing to sufficiently stabilize dystrophic skeletal muscles. Supporting this hypothesis, the heart (which is believed to be at least partially protected from turnover) demonstrates increased dystrophin correction and expression over time. Based on these observations we investigated co-delivery of microdystrophin with CRISPR/Cas9 to examine if microdystrophin could serve to stabilize skeletal muscles and provide an extended time frame for enhancing gene-correction in the absence of muscle turnover. Here we present *in vivo* approaches for muscle-specific systemic correction of the dystrophin gene in the *mdx*^{4cv} mouse model of DMD using multiple AAV vectors carrying microdystrophin, Cas9 and sgRNA cassettes. While much optimization still needs to be done to ensure effectiveness and safety, AAV-mediated muscle-specific gene editing shows significant potential for correction of mutations leading to DMD and other genetic muscle disorders.

860. A Design of the Quantitative Evaluation Method for Cognitive Impairment in Mdx Mouse

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Duchenne muscular dystrophy (DMD) is a progressive muscular degenerative disorder, caused by mutations in the dystrophin gene. Dystrophin and components of the dystrophin-associated glycoprotein complex (DGC) are located at the plasma membrane and play an important role of muscular stability. The loss of dystrophin and DGC lead to muscle inflammation and degeneration, resulting in muscle waste and physical disability. While in the central nervous

system (CNS), these proteins are located at postsynaptic membrane of GABAergic synapses and contribute to cognitive function. Therefor impairment in CNS due to the lack of dystrophin and DGC have also been described in one third of DMD patients, including autism spectrum disorder, mental retardation and poor academic performance. Dystrophin is expressed in brain tissue, such as cerebellar Purkinje cells, amygdalae and hippocampal neurons in mouse. In dystrophin-deficient *mdx* mouse, which is model mouse with DMD, deficit of dystrophin and DGC in neurons leads to cognitive dysfunction. Microdystrophin therapy with adeno-associated virus (AAV) for *mdx* mouse has been shown to reduce skeletal muscle disease and cardiomyopathy. Meanwhile, it is unclear whether cognitive dysfunction is improved or not. To assess cognitive function and to evaluate microdystrophin therapy in the brain, we designed the quantitative evaluation methods using a combination of behavioural test and recording of spontaneous locomotor activity. We injected 1e+13 vector genome per kilogram of AAV9-CMV-microdystrophin via retro orbital vein to 3 days-old male *mdx* mouse. At 8 weeks-old, we performed behavioural test and recording of spontaneous locomotor activity. Western blot analysis confirmed dystrophin expression in brain tissue at 10 weeks after gene transfer. *Mdx* with AAV injection showed significantly reduce freezing time as behavioural test. Our results suggest that microdystrophin therapy improved cognitive dysfunction in *mdx* mouse. This observation methods may be useful in evaluating of *mdx* mouse compared with wild type or AAV injection *mdx* mouse in behavior analysis and cognitive function.

861. AAV-9 CRISPR Mediated Satellite Cell Editing Restored Dystrophin Expression after Complete Degeneration in a Whole Muscle Graft Model

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Abstract. Adeno-associated virus serotype 9 (AAV-9) mediated CRISPR-Cas9 gene editing is a promising approach to restore missing dystrophin for Duchenne muscular dystrophy (DMD) therapy. A permanent therapy likely depends on efficient repairing of satellite cells which are the stem cells responsible for muscle repair/regeneration throughout adult life. It is hotly debated whether AAV-9 CRISPR delivery can edit satellite cells. We addressed this issue using a whole muscle graft model. Specifically, the tibialis anterior (TA) muscle from the donor was grafted to the surgically cleared TA compartment in an immune deficient host mouse. The donor muscle undergoes complete necrosis followed by regeneration from satellite cells. We first determined the relative contribution of the donor and host satellite cells to the formation of nascent myofibers in the graft. Using cross-transplantation between GFP+ and GFP- donor/host pairs, we found that ~97% regenerated myofibers were derived from the donor. However, traces of host myogenic cells were observed within TA grafts. Next, to investigate satellite cell editing, we treated 6-week-old dystrophin-null mdx mice with AAV-9 CRISPR via tail vein injection. 18 months later, we grafted the TA muscle to immune-deficient dystrophin-null host. AAV-9 CRISPR therapy resulted in 22.1%±13.0 dystrophin positive myofibers in the mdx TA muscle prior to graft. Following grafting, the regenerated muscle contained 4.6%±2.7 dystrophin positive fibers. As a control, we also grafted the TA muscle of age-matched mdx mice that were not treated by AAV-9 CRISPR. Prior to the grafting, there were 1.4%±0.5 dystrophin positive cells. In regenerated grafts, this number was 1.7%±1.2, significantly lower than what was observed in AAV-9 CRISPR treated donors. Subsequent genomic sequencing confirmed the presence of the edited genome only in the grafts derived from AAV-9 CRISPR treated donors. Our data suggest that AAV-9 CRISPR delivery indeed resulted in satellite cell editing. Strategies that can improve the efficiency may have important implications for DMD gene repair therapy (supported by NIH and DOD).

862. Isolated Limb Infusion of rAAVrh74.MCK.*GALGT2* in the Rhesus Macaque Allows for Broad Transgene and CT Glycan Expression in Limb Skeletal Muscles

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rAAV vectors require high doses for systemic treatment of cardiac and skeletal muscles, the target tissues in muscular dystrophy. In some instances, isolated limb infusion may allow for lower doses of vector to be used in a manner that reduces overall organ toxicity. Here we report on an isolated limb infusion technique in a non-human primate where hindlimb blood flow is transiently isolated during vector delivery to concentrate vector in targeted leg muscles. A bilateral dose of $2.5x10^{13}$ vector genomes (vg)/kg/limb of rAAVrh74.MCK.GALGT2 was sufficient to induce GALGT2 expression in 10-60% of skeletal myofibers in all leg muscles. Muscles from a limb receiving isolated limb infusion had, on average, a 12±3-fold increase in vg compared to the same muscles in an untreated limb after vector was released into the systemic circulation. Variability in AAV biodistribution between different segments of the same muscle, however, was high, suggesting limitations to uniform transduction. These results demonstrate that treatment of muscles throughout the leg with rAAVrh74.MCK.*GALGT2* can be accomplished safely using an isolated limb infusion technique where balloon catheters transiently isolate the limb vasculature.

863. Lentiviral Gene Therapy for Bone Repair Using Human Umbilical Cord Blood Derived-Mesenchymal Stem Cells

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Introduction: There is great interest in using umbilical cord blood (UCB) as a source of multipotent stem cells for use in regenerative medicine because of the need for less stringent HLA matching. Allogeneic UCB-MSCs combined with an osteoinductive growth factor could be used as an off-the-shelf product and thus revolutionize the treatment of challenging bone defects. In this study we aimed to develop an ex vivo gene therapy strategy using BMP-2 transduced allogeneic UCB-MSCs to promote bone repair. The goals of this study were to a) test the ability of a lentiviral vector (LV) carrying the cDNA for bone morphogenetic protein 2 (BMP-2) to successfully transduce human UCB-MSCs, leading to BMP production and osteogenic differentiation *in vitro* and b) assess the capacity of BMP-2 transduced UCB-MSCs to induce heterotopic bone formation in a mouse muscle pouch. **Methods:** Human UCB-MSCs isolated from full-term deliveries cord blood were expanded in culture using standard protocols. UCB-MSCs were transduced overnight with a LV overexpressing BMP-2 or GFP (MOI=25) and assessed in vitro for transduction efficiency, osteogenic differentiation and BMP-2 production. The in vivo ostegenic potential of UCB-MSCs was tested in a hind limb muscle pouch model in NSG mice. The following groups were assessed: UCB-MSCs transduced with LV-BMP-2 (Group I); LV-GFP (Group II); and non-transduced UCB-MSCs (Group III). Bone formation was evaluated with radiographs, histology and histomorphometry (Bone area-BA, Tissue area-TA) at 4 weeks. **Results:** FACS analysis of GFP-transduced UCB-MSCs confirmed successful transduction, with a transduction efficiency of 97%.(Figure 1A) Transduction with LV-BMP-2 was associated with abundant BMP-2 production (40.5±18.8ng/24h/10^6 cells) (Figure 1B) and induction of osteogenic differentiation *in vitro*. Implantation of BMP-2 transduced UCB-MSCs in NSG mice led to robust bone formation at 2 and 4 weeks post-operatively as seen on radiographs. (Figure 2) Histology revealed formation of woven bone with reconstitution of the bone marrow cavity in the muscle pouch 4 weeks after implantation with UCB/LV-BMP-2. In contrast, no evidence of ectopic bone formation was noted in animals treated with GFP- or non-transduced UCB-MSCs.(Figure 2) Histomorphometric analysis confirmed the differences between the groups in bone formation, with the BMP-2 implanted animals demonstrating a significantly higher mean BA and BA/TA compared to the control groups ($p<0.001$). **Conclusion**: Human MSCs derived from UCB can be transduced successfully with a LV overexpressing BMP-2, leading to abundant BMP production and induction of osteogenic differentiation in vitro, as well as heterotopic bone formation when implanted into a mouse model. These results, along with the fact that UCB-MSCs can be easily collected with no donor-site morbidity and low immunogenicity, suggest that UCB might be a preferable allogeneic source of MSCs to develop an ex vivo gene therapy approach to treat difficult bone repair scenarios.

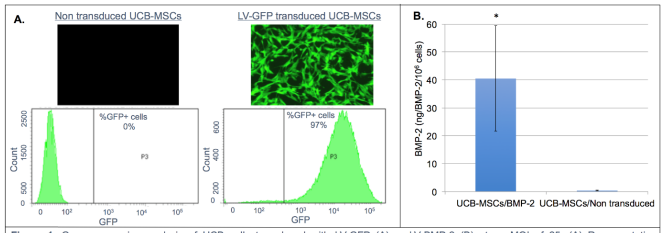

Figure 1. Gene expression analysis of UCB cells transduced with LV-GFP (A) or LV-BMP-2 (B) at an MOI of 25. (A) Representative photomicrographs of GFP expression and FACS analysis in non-transduced and GFP-transduced UCB-MSCs 48h post-transduction. (B) In vitro BMP-2 production by UCB-MSCs transduced with LV-BMP-2. The results are presented as ng of BMP-2/24 hours per 1x10^6 cells. Non-transduced cells were used as negative control. *p<0.05 compared with non transduced cells.

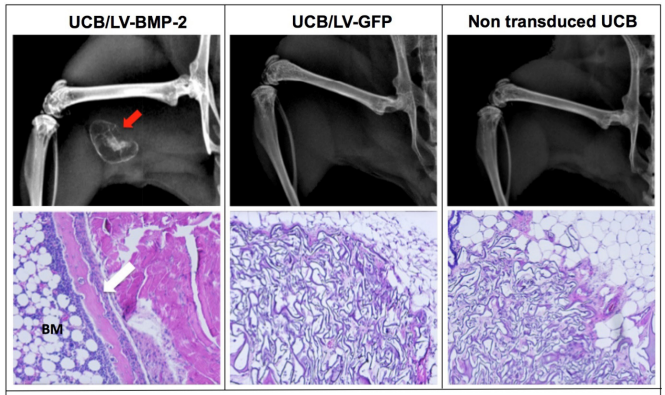

Figure 2. Representative radiographic and H&E histologic images 4 weeks following implantation. Abundant bone formation is noted in the muscle pouch implanted with UCB/LV-BMP-2 cells (red arrow), with mature osseous components, including woven bone and bone marrow In contrast no bone formation is seen in the animals treated with GFP-transduced or non-transduced UCB cells.

864. Biological Effects of FGF-2 and IGF-I Co-Overexpression in Human Bone Marrow-Derived Mesenchymal Stem Cells via rAAV Vector Administration

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Introduction: Gene-based modification of bone marrow-derived mesenchymal stem cells (MSCs)is an attractive approach to treat articular cartilage defects. Here, we tested the benefits of co-delivering rAAV-FGF-2 and rAAV-IGF-I in human MSCs as a tool to enhance the cartilage repair. **Methods:** rAAV-lacZ carries the E. coli β -galactosidase (lacZ) gene, rAAV-hFGF-2 a human FGF-2 sequence, and rAAV-hIGF-I a human IGF-I sequence, all controlled by the CMV-IE promoter/enhancer[1,2]. Bone marrow aspirates were obtained from the distal femurs of donors undergoing total knee arthroplasty, washed, and MSCs we prepared as previously described[1,2].Cells were (co)transduced with rAAV (FGF-2 + IGF-I: 40µ l each vector; lacZ: 40µl l vector) and kept in chondrogenic medium for up to 21 days. Histological and immunohistochemical analyses were performed on paraffin-embedded sections of the constructs (5µm) (toluidine blue staining; anti-FGF-2, anti- IGF-I, and anti-type-II/-I/-X collagen immunostaining)[1,2]. The proteoglycan contents were monitored by binding to dimethylmethylene blue dye and the DNA contents by Hoechst 33258 assay[1,2]. Total RNA was extracted and reverse transcription carried out for cDNA amplification via real-time RT-

PCR with GAPDH as control for normalization[1,2]. Each condition was performed in duplicate in three independent experiments. A t-test was employed with $p \leq 0.05$ considered statistically significant. Results and Conclusion: **Results:** Successful overexpression of the two candidate growth factors was observed in hMSC aggregates upon co-delivery of the rAAV vectors coding for FGF-2 and IGF-I compared with the control (*lacZ*) condition over the period of evaluation (Fig. 1). Chondrogenic differentiation was evidenced in the aggregates after 21 days, especially using cells cotransduced with FGF-2 and IGF-I as seen by intense toluidine blue staining and type-II collagen immunostaining (Fig. 2). Co-application of FGF-2 with IGF-I significantly increased the proteoglycan contents in the aggregates relative to the control treatment (2-fold difference; $p \leq 0.001$), while no effects were noted on the DNA contents in the conditions employed here (Table 1). Remarkably, immunoreactivity to type-I and -X collagen was less intense with the two FGF-2 and IGF-I candidate vectors (Fig. 2). These findings were corroborated by the results of a real-time RT-PCR analysis revealing enhanced chondrogenic differentiation with FGF-2 and IGF-I *versus* control treatment (3-fold difference in COL2A1 expression; $p \leq 0.001$) and reduced hypertrophic differentiation (up to 2- and 2.5 -fold difference in COL1A1 and COL10A1 expression; $p \leq 0.001$), probably due to increased SOX9 levels (3-fold difference; $p \leq 0.001$) (Fig. 3). **Conclusion:** Significant, sustained cooverexpression of FGF-2 with IGF-I in hMSC aggregates upon co-transduction via rAAV is capable of stimulating the chondrogenic activities to treat cartilage defects. **References:** [1] Cucchiarini *et al.*, *Tissue Eng Part A* **2011**, 17:1921; [2] Frisch *et al.*, *Stem Cell Res Ther* **2014**, 5:103. ACKNOWLEDGMENTS: Work funded by the German Research Society.

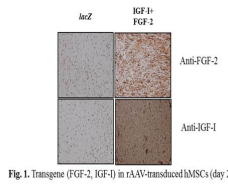

Fig. 1. Transgene (FGF-2, IGF-I) in rAAV-transduced hMSCs (day 21).

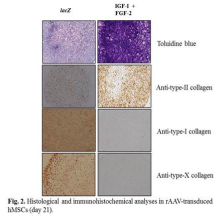

Fig. 2. Histological and immunohistochemical analyses in rAAV-transduced hMSCs (day 21).

Table 1. Biochemical analyses (day 21).

Treatment	PGs (µg/pellet)	DNA (ng/pellet)	PGs/DNA (µg/ng)
lacZ	0.9 (0.1)	26.0 (0.4)	0.1 (0.1)
FGF-2 + IGF-I	1.2 (0.1)*	25.0 (0.2)	0.2 (0.1)*

Data are given as mean (SD). PGs: proteoglycans. *Statistically significant compared with *lacZ*.

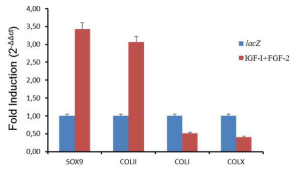

Fig. 3. Real-time RT-PCR analysis in rAAV-transduced hMSCs (day 21).

865. Next-Generation Human Artificial Chromosomes for Autologous Cell Therapy of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is an incurable neuromuscular disorder caused by mutations in the largest human gene: dystrophin (cDNA 14 kb, genetic locus 2.4 Mb). Transferring the dystrophin gene or cDNA into primary human stem/progenitor cells is limited by restrictions in the cargo ability of conventional gene therapy vectors, posing hurdles to the success of autologous cell therapy protocols for DMD. The use of large-capacity vectors, such as human artificial chromosomes (HACs), as a system of gene delivery may overcome this limitation. We previously reported morphological and functional amelioration of a DMD mouse model transplanted with dystrophic murine muscle progenitors genetically-corrected with a HAC containing the entire dystrophin locus (DYS-HAC). However, in order to apply HAC-mediated gene-correction to human muscle progenitors, an extension of their proliferative potential will be required to withstand clonal cell expansion after HAC transfer. Here, we show that reversible cell immortalisation mediated by lentivirally delivered excisable hTERT and Bmi1 transgenes increased human muscle progenitor cell proliferation. The extended proliferation allowed the transfer of a novel DYS-HAC into DMD satellite cell derived myoblasts and perivascular cell-derived mesoangioblasts, two muscle progenitor cell types which have already undergone clinical experimentation. HAC-genetically corrected cells did not undergo tumorigenic transformation, maintained a stable karyotype and their migration ability. Cells also retained their myogenic ability in vitro and engrafted murine skeletal muscle upon transplantation in mice. Lastly, we engineered a next-generation HAC capable of delivering i) complete genetic correction, ii) reversible immortalisation, iii) additional dystrophin expression, iv) inducible differentiation and v)

controllable cell death. Taken together this results describes a novel strategy for complex gene transfer into human muscle progenitors and the development of next-generation multifunctional artificial chromosomes for DMD autologous cell therapy.

866. CRISPR-Induced Deletion for the Correction of the Human Dystrophin Gene Using the Cas9 from *S. aureus*

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Neurosciences axis, Centre de Recherche du CHU de Québec, Québec, QC, Canada

Duchenne muscular dystrophy is a severe genetic disease, affecting 1/5000 newborn boys, characterized by muscle wasting and weakening that lead to cardio-respiratory failure and to death in the twenties. This X-linked disease is related to a mutation (mostly deletion of one or several exons) in the DMD gene (coding for dystrophin) that is responsible of a shift in the reading frame thus creating a premature stop codon that abrogates dystrophin protein synthesis. We report that by using a combination of sgRNAs recognized by the SaCas9 (*S. aureus),* we can create a large genomic deletion that reframes the dystrophin gene in myoblasts from DMD patients and allows expression of dystrophin in myotubes. Our approach differs from others as in addition to restore the expression of the dystrophin protein, we also aim to restore the correct structure of the spectrin-like repeat (SLR) in its central rod-domain as observed in patients with Becker muscular dystrophy, who do not have a severe phenotype, because their internally deleted dystrophin protein is functioning normally. Following the screening of sgRNAs targeting the exons 46 to 58 of the DMD gene, we identified pairs of sgRNAs able to form hybrid exons 46-51, 46-53, 47-52, 49-52, 49-53, and 47-58 in 293T cells. In these hybrid exons, the nucleotide sequences were exactly as expected in 54% to 90% of the amplicons, according to TIDE analysis. Interestingly, two pairs of sgRNAs generated large genomic deletions connecting the exon 47 to the exon 58 that might correct up to 40% of the DMD mutations. In addition, the resulting amino-acid sequences of such hybrid exons have been modeled by iTasser. The resulting models indicated that the spectrin-like repeat structure (SLR) of these internally deleted dystrophin proteins was perfect. These pairs of sgRNAs were thus tested in myoblasts of 4 different DMD patients and we detected the formation of the hybrid exons. Deep sequencing indicated that 40% to 89% of these hybrid exons contained the exact expected nucleotide sequences. Moreover, after formation of myotubes by the myoblasts containing a hybrid exon, the truncated dystrophin protein was detected. Preliminary work in the hDMD/mdx mouse model demonstrated the *in vivo* feasibility of our approach since the formation of the hybrid exons 47-58 was observed in the *Tibialis anterior*, the heart and the diaphragm, following systemic administration of two AAV9s ($1.5.10^{12}$ v.g.) respectively coding for SaCas9 and for a pair of sgRNAs. Experiments aiming to demonstrate the restoration of the dystrophin protein expression in a new $hDMD^{del52}$/mdx mouse model are under way. This work supports the feasibility of creating a large genomic deletion permitting the production of an internally-truncated dystrophin protein that could lead to functional improvement in such model.

867. Delivery of CRISPR/Cas9 via Viral and Non-Viral Methods to Reframe the *DMD* Gene in a Humanized Mouse Model of Duchenne Muscular Dystrophy

Courtney S. Young[1], Ying Ji[2], Xiangsheng Liu[2], Ekaterina Mokhonova[3], Michael Emami[1], Niclas Bengtsson[4], Jeffrey S. Chamberlain[4], April D. Pyle[5], Huan Meng[2], Melissa J. Spencer[3]

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Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disorder caused by out-of-frame mutations in *DMD,* which prevent dystrophin production. Some therapeutic strategies are designed to reframe the *DMD* gene, thereby turning Duchenne into the clinically milder allelic disease, Becker muscular dystrophy. We have developed a CRISPR/Cas9 platform to reframe *DMD* using a single pair of guide RNAs to delete exons 45-55, which in patients leads to one of the mildest Becker phenotypes. This region also encompasses a hotspot of ~50% of all DMD patient mutations. Here we have applied this platform *in vivo* to our novel humanized dystrophic mouse model, which contains an out-of-frame mutation in a transgenic human *DMD* gene. We used both viral and non-viral methods to deliver our CRISPR/Cas9 platform to muscle *in vivo*. We demonstrated restored dystrophin protein in skeletal muscle after local and systemic injections of AAV6 versus a polymer nanoparticle carrier. This work demonstrates the potential for systemic delivery of a single CRISPR/Cas9 platform to restore dystrophin for half of DMD patients.

868. Complementary Techniques to Evaluate Microdystrophin Expression in Duchenne Muscular Dystrophy Gene Therapy Studies

Brittany Fickau[1], Margaret Beatka[1], Patrick Gonzales[2], Hui Meng[1], Sharla Birch[3], Carl A. Morris[2], Samuel Ayres[1], Jennifer Tinklenberg[1], Mark Vanden Avond[1], Joe Kornegay[3], Joel S. Schneider[2], Kristy J. Brown[2], Michael W. Lawlor[1]

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Duchenne muscular dystrophy (DMD) is a debilitating, degenerative muscle condition that is pathologically characterized by the near-absence of dystrophin in skeletal muscle and evidence of recurrent myofiber degeneration and regeneration. A recent focus of treatment is the use of adeno-associated virus (AAV) mediated gene transfer to induce expression of a shortened form of dystrophin (microdystrophin) in muscle to reduce disease severity. With such a treatment approach, the evaluation of microdystrophin expression in muscle is of critical importance to assess safety and efficacy across different preclinical animal models. Using multiple complementary techniques such as immunofluorescence and western blot, we have evaluated the

expression of microdystrophin in *mdx* mice and golden retriever muscular dystrophy (GRMD) dogs across a variety of treatment paradigms. Tissues from these studies provided an opportunity to further refine methodology for the assessment of microdystrophin in muscle. Data presented will focus on illustrating key areas of improvement.

869. *In Silico* Platform for the Design and Generation of Novel Muscle Promoters: *In Vitro* Validation

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Gene therapy-mediated transgene activity is driven by specialized DNA sequences that direct protein expression in either a ubiquitous or tissue-specific fashion. These sequences, termed promoters, can be derived from either naturally occurring endogenous sequences or synthetically designed to accommodate certain parameters such as tissue specificity, viral packaging limitations or to drive varied levels of protein expression. In this study, a panel of *in silico* designed synthetic promoters were generated and assessed for their ability to drive high levels of skeletal muscle-specific protein expression. Bioinformatics analysis of multiple genomic datasets (including gene expression and epigenetics data) was used to identify candidate enhancer and core promoter regions active in a set of genes differentially over-expressed in muscle. The bioinformatics analysis was done using PromPT®, a proprietary bioinformatics platform optimized for the identification of genomic regulatory regions. Candidate enhancer elements were synthesized upstream of the core promoter and transfected into skeletal muscle cells. Promoter activity was assessed in differentiated myotubes. Enhancer elements were then combined to create novel muscle specific synthetic promoters. Multiple candidate synthetic promoters were shown to have higher activity than CMV, a ubiquitous promoter, in differentiated rodent and human skeletal muscle cells. Importantly, these promoters demonstrated high tissue specificity, with minimal expression in non-muscle cell types, including kidney and liver cells. These data suggest that entirely novel regulatory sequences can be derived from computational analyses and tailor-suited to specific pre-designated requirements.

870. Proteomic Profiling in Nemaline Myopathy to Identify Disease Subclass Biomarkers

Emily Siebers[1], Jennifer Tinklenberg[1], Hui Meng[1], Samuel Ayres[1], Mark Vanden Avond[1], Rebecca Slick[1], Kristen Nowak[2], Henk Granzier[3], Edna Hardeman[4], Federica Montanaro[5], Michael Lawlor[1]

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Nemaline myopathy (NM) is a muscle disorder that can cause death or lifelong disability. NM is clinically and genetically heterogeneous, and it is only considered a single disease entity due to the presence of nemaline rods on muscle biopsy. While nemaline rods are diagnostically helpful, their presence does not correlate well with disease severity, and their contribution to the weakness seen in NM is questionable. Where subclassification of NM patients is attempted, distinctions are often made based on the causative gene, but this does not predict patient prognosis well. We have recently worked with the *Acta1* H40Y, *Acta1* D286G, and *Neb* cKO mouse models of NM, and we have observed considerable variation in disease biology and the impact of anti-myostatin therapy across these models. We hypothesize that as-yet-unappreciated biological processes play a role in the weakness of NM, and that biomarkers related to these processes can be used to relate subsets of NM patients to existing animal models. We have performed proteomic analysis of the *Acta1* H40Y model of NM to identify differences in protein expression in comparison to their wild type (WT) counterparts. Based on our findings, we interrogated immunohistochemical biomarkers (including proteins with the highest degree of overexpression in *Acta1* H40Y mice, and those involved with interesting potential mechanisms of weakness) in frozen skeletal muscle samples from *Acta1* H40Y, *Acta1* D286G, and *Neb* cKO mouse models. Interestingly, several of these markers (Tmod1, Coronin 6, Pascin 3) were present in the pathological aggregates of *Acta1* H40Y tissue but were not observed in pathological aggregates in the other two NM models. Current work is focused on evaluating the expression of these proteins in human NM muscle samples, in addition to performing similar proteomics analyses using tissue from *Acta1* D286G, and *Neb* cKO mice at various disease stages. Our work will help correlate biochemical abnormalities to weakness and pathology across NM models and will be used to develop new strategies of biomarker evaluation and disease classification in NM.

Neurologic Diseases (Including Ophthalmic and Auditory Diseases) III

871. Strategies to Enhance and Modulate Hematopoietic Stem Cell Contribution to Brain Myeloid Cell/Microglia Turnover

Rita Milazzo*[1], Francesca Ferro*[1,2], Annita Montepeloso[2], Eleonora Cavalca[1,2], Valentina Cipolla[2], Hui Yu[2], Sabyasachi Das[2], Janki Patel[2], Danilo Pellin[2], Marco Peviani[1,2], Alessandra Biffi[1,2,3]

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Our recent data indicate that the delivery of hematopoietic and progenitor stem cells (HSCs) in the brain lateral ventricles (intracerebral ventricular - ICV - delivery) of conditioned recipient results in a rapid, robust and central nervous system (CNS)-restricted engraftment of transplant-derived myeloid cells sharing functional features of microglia. This repopulation is driven by early homing after transplant and long term engraftment of HSCs in the brain that generate a myeloid local progeny (Capotondo, Milazzo et al., Sci. Advances 2017). The ICV delivery route *per se* has therapeutic relevance because it allows for delivery of therapeutic molecules to the brain and modulation of microglia function for the treatment of disorders affecting the CNS. Moreover, it also offers the opportunity of enhancing and fastening brain engraftment and therapeutic benefit of a standard HSPC transplantation strategy in the context of a combined approach. In order to develop this strategy toward the clinics we tested different combinatorial HSC based transplant protocols modulating the contribution of intravenous (IV) *versus* intracerebroventricular (ICV) transplanted cells to the CNS and to medullar hemapoiesis in terms of population employed at each site and timing of cell delivery. In the context of this study, we could characterize and fate map the CNS-progeny of the transplanted cells and obtain relevant insights on the mechanism of HSC engraftment and differentiation in the CNS. Indeed, we observed that the route of cell delivery determines i) a different CNS microglial and medullar hematopoietic reconstituting potential of the transplanted HSCs, ii) a specific pattern of distribution of the transplanted cell progeny in the brain and iii) a differential stage of maturation of IV versus ICV-derived cells, as shown by morphological and transcriptional studies. Importantly, we documented the unique role of the ICV delivery route for a not only fostered myeloid engraftment of the transplanted HSCs, but also their more efficacious differentiation into morphologically- and functionally-defined microglia cells distributed throughout the CNS. Notably, the delivery of cells ICV also favored and positively affected the maturation of IV co-delivered cells. Overall, our results demonstrate the biological relevance of HSC transplantation within the CNS. Its therapeutic potential was also shown in neurodegenerative disease animal models in the context of autologous gene therapy and allogeneic transplantation.

872. OXB-201 (Retinostat®), a Lentiviral Vector Expressing Endostatin and Angiostatin, is Safe, Well Tolerated and Results in Stable Protein Expression for More Than 5 Years in Patients with Advanced Neovascular Age-Related Macular Degeneration

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Introduction Age-related macular degeneration (AMD) is one of the most common causes of vision loss and leads to impaired vision in approximately 1.7 million people in the USA alone. The "wet" form involves leakage from pathological neovascularization under the retina resulting in oedema and progressive vision loss. High levels of vascular endothelial growth factor (VEGF) in the oedematous retina promote further angiogenesis, thus current treatments involve repeated monthly intravitreal injections of anti-VEGFs. An alternative treatment that avoids the complications, discomfort and healthcare burden associated with repeat injections is under investigation. OXB-201 is engineered from the Equine Infectious Anaemia Virus (EIAV) lentivirus expressing human endostatin and human angiostatin, both potent anti-angiogenic proteins. OXB-201 is delivered via subretinal injection with the aim of delivering long-term production of the therapeutic proteins. **Methods** A phase I dose-escalation safety study (GEM) was performed in patients with advanced neovascular age-related macular degeneration. Following the administration of a single subretinal injection of OXB-201, patients were assessed on day 1, and at various time points between weeks 1 and 48. Following the completion of the GEM study, patients were enrolled onto a long-term safety study (GEM LFTU) in which patients are evaluated every 6 months for the first 5 years and annually for the next 10 years. Throughout both trials, assessments have included various ophthalmological examinations, biodistribution and expression of the human transgenes endostatin and angiostatin in aqueous fluid taps. **Results** Of 21 patients that received a subretinal injection of OXB-201, expression of the transgenes endostatin and angiostatin were detectable in 20 and 21 patients, respectively. Protein expression was detected as early as week 1, reached a peak by week 12 and thereafter plateaued. The mean fold difference in protein expression levels between the maximum and minimum levels of endostatin and angiostatin detected between week 12 (GEM) and the most recent assessment time point (GEM LFTU out to 24-60 months) in the higher dose groups (cohorts 2-4) was 3-fold and 2.2-fold, respectively. The maximum fold difference in protein levels in an individual patient was 7-fold for endostatin and 3.3-fold for angiostatin. Biodistribution studies were negative. **Conclusions** Subretinal administration of escalating doses of OXB-201, the first ever lentiviral vector directly administrated to the eye, has been shown to be safe, well tolerated. Following treatment with OXB-201, reductions in active leakage at all dose levels was seen which were associated with the expression levels of endostatin and angiostatin. Remarkably expression of endostatin and angiostatin has been extremely stable and persistent throughout

(GEM+LFTU: total of 6 years so far). Given the benign safety profile and the long-term expression of the therapeutic proteins (the first time gene expression has been measured in a clinical trial), the EIAV LentiVector® platform has demonstrated the requirements to be an outstanding gene therapy platform to treat human conditions.

873. Intravenously Administered Gene Therapy for Neuronopathic Gaucher Disease

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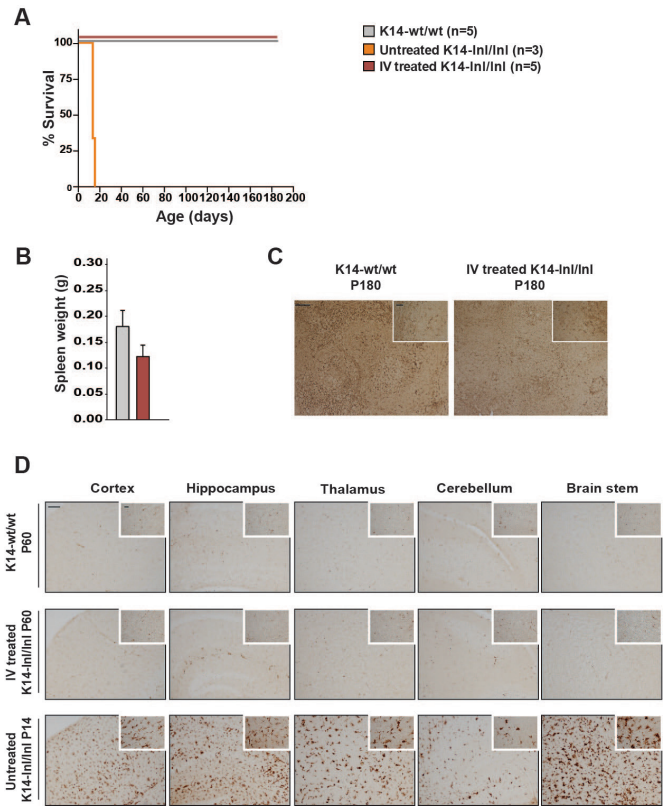
Gaucher Disease (GD) is a lysosomal storage disorder caused by mutations in the *GBA1* gene encoding the enzyme glucocerebrosidase (GCase). Deficiency of glucocerebrosidase causes the accumulation of its substrate glucosylceramide in visceral organs and the brain. Enzyme replacement therapy is successfully used to ameliorate the visceral pathology, however there is no treatment available for the lethal neurodegeneration. This research focuses on Gaucher disease type II, the most acute neuronopathic form, in which neuropathology results in death in early infancy. The aim of the project is to intravenously administer an adeno-associated viral vector serotype 9 (AAV9) carrying the functional *GBA1* gene to a GCase-deficient mouse model of GD type II and assess improvement in lifespan, behavior, brain and visceral pathology. Different AAV9 vectors carrying the functional human *GBA1* gene were intravenously administered into neonatal knock-out mice and compared. The untreated knock-out mice die 12-14 days after birth from acute neurodegeneration. Treated animals showed significant increase in their lifespan (A) and the neuropathology was ameliorated (D). Spleen weight (B), histological analysis (C), enzymatic assay and blood test revealed improvements in the visceral pathology. Further optimization of the vector has subsequently been carried out in order to enhance the therapeutic effects of the treatment. This minimally invasive pre-clinical proof-of-concept gene therapy study supports potential clinical translation for the acute early-lethal neuronopathic form of Gaucher disease, for which therapeutic needs are currently unmet.



874. Improvement of Motor and Cognitive Function by Gene Therapy for Patients with AADC Deficiency

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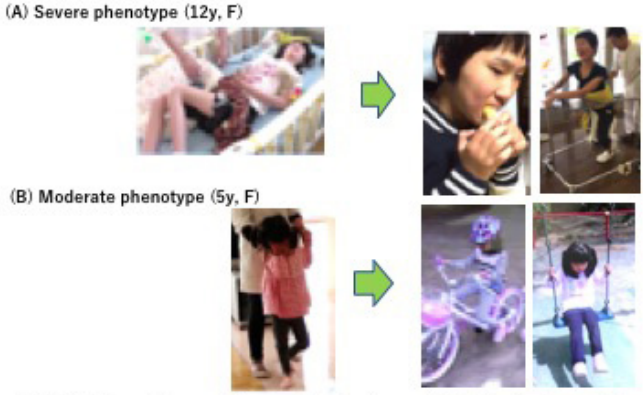
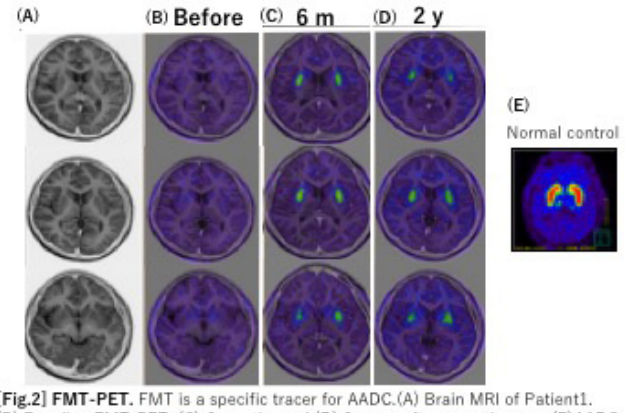
Patients with aromatic l-amino acid decarboxylase (AADC) deficiency cannot produce dopamine, catecholamines and serotonin in the brain. They exhibit oculogyric crises, dystonia, impaired voluntary movements, intellectual disability and autonomic dysfunction. Many patients were bedridden for entire life. We report the clinical course of five severe patients with AADC deficiency and one moderate patient whose cognitive and motor function improved markedly after gene therapy by using AAV2 vector. **Patients and Methods**: Three male (4, 10, 15 and 19year-old) and 1 female (12year-old) patients with severe phenotypes were bedridden with no voluntary movements nor speech. One female patient (5year-old) with moderate phenotype could walk with support and speak several words after MAO-B inhibitor treatment. They received 2×10^{11} vector genomes of AAV2 vector harboring *AADC* gene via bilateral intraputaminal infusions by stereotactic neurosurgery. **Results:** By two years after gene therapy, all patients showed an improved motor function and no dystonia. Two severe patients walked with a walker, and the moderate patient could run and ride a bicycle. [FIg.1] "$$graphic1" Regarding mental development, the moderate

phenotype patient could converse. The 12-year-old female with severe phenotype responded quickly to spoken orders. All patients exhibited transient chorea as adverse events. Positron emission tomography with 6-[^{18}F]fluoro-l-*m*-tyrosine, a tracer of AADC, showed persistent *AADC* expression after gene therapy. [Fig.2] "$$graphic2" **Discussion**: Since the putamen is the main output structure of the motor network in the basal ganglia, improved motor performance and amelioration of dystonia can be expected after putaminal AADC gene delivery. The motor, cognitive and speech functions remarkably improved in the moderate patient. The restoration of dopamine production in the putamen may have broader effects depending on the patient age at treatment and genetic severity.



[Fig.1] Pictures of severe & moderate phenotype patients (before therapy & post 2yrs). **(A)**Before therapy, she was bedridden without voluntary movement. 2yrs after, she can walk with walker and eat orally. **(B)**Before therapy, she could walk with support. 2yrs after, she can ride a bicycle and play with a swing.



[Fig.2] FMT-PET. FMT is a specific tracer for AADC.(A) Brain MRI of Patient1. (B) Baseline FMT-PET, (C) 6 months and (D) 2 years after gene therapy. (E)AADC was detected in putamen of normal healthy control. In brain of AADC deficiency, AADC were not detected before gene therapy(B).After GT, AADC expression remaind up to two years.

875. Complement Mediates NLRP3-dependent IL-1β Secretion in Experimental Autoimmune Uveitis and Its Inhibition by AAV Based Gene Delivery of Soluble CD59

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Purpose: Complement Membrane attack complex (MAC) is a critical terminal component of the innate immune system and its role has been described in various inflammatory diseases. Here, we investigate the role of the MAC mediated NLRP3-dependent IL-1β secretion in experimental autoimmune uveitis (EAU). Furthermore, we investigate adeno-associated virus (AAV) based gene delivery of sCD59 (protectin) for inhibition of MAC triggered NLRP3-dependent IL-1β secretion and rescue of EAU pathology in the mouse retina. **Methods:** EAU was induced in C57Bl/6J and C9$^{-/-}$ mice. After a period of 24 days, eyes were enucleated and immunohistochemistry was performed in retinal cryostat sections for quantification of MAC deposition. The levels of cytokine release including IL-1β, IFN-γ, and IL-17 were analyzed by ELISA and qPCR in retinas. NLRP3, Caspase 1 and ASC protein expression was measured by western blot. Electroretinogram (ERG) recordings were done to analyze the retinal function and clinical changes were quantified by fundus imaging and histology respectively. Similarly, these parameters were measured in mice that had been pre-injected with either a control GFP-expressing AAV vector (AAVCAGGFP) or sCD59 (AAVCAGsCD59) - a vector currently in Phase I for the treatment of dry age-related macular degeneration. **Results:** The EAU retina had 70% increased MAC deposition and two-fold increased activation of NLRP3 protein and 80% more Caspase 1 activation compared to normal C57Bl/6J retina. We found increased levels of IL-1β, IFN-γ and IL-17 cytokines in EAU retina relative to normal retina. The absence of MAC in C9$^{-/-}$ EAU retinas resulted in two-fold inhibition of NLRP3 protein and 50% inhibition of Caspase 1 activation and prevented secretion of IL-1β and IFN-γ. However, EAU-associated clinical symptoms and pathology including retinal function were not rescued in C9$^{-/-}$ mice. An intravitreal injection of AAVCAGsCD59 in EAU mice inhibited MAC deposition by 45% and NLRP3 protein and Caspase 1 activation by 60% along with inhibition of IL-1β, IFN-γ, and IL-17 relative to controls. ERG recordings from AAVCAGsCD59 treated retinas showed more than 40% improvement in retinal function relative to AAVCAGGFP treated control retinas. Further, AAVCAGsCD59 treated retinas had significantly lower clinical and histological scores relative to control retinas. **Conclusions:** MAC regulates NLRP3-dependent IL-1β secretion and differentiation of T cells in the development of EAU. AAV mediated gene delivery of soluble sCD59 has long-term potential in treating uveitis.

876. Dose-Response and Stability Study of AAV4CAGhTPP1 in a CLN2$^{-/-}$ Rodent Model of Late Infantile Neuronal Ceroid Lipofuscinosis

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Late infantile Neuronal Ceroid Lipofuscinosis (LINCL) is a childhood neurodegenerative disease that appears between 2-4 years of age and progresses with visual, motor, and mental decline, with a life expectancy of less than two decades. LINCL is caused by deficiency in the soluble lysosomal enzyme TPP1 as a result of mutations in the *CLN2* gene. TPP1 is a mannose-6-phosphate decorated enzyme which makes it amenable to a cross-correction approach by gene therapy. In this study, the human *CLN2* gene was introduced by adeno-associated virus into the ependymal cells lining the brain ventricles of TPP1 deficient mice. AAV4CAGhTPP1 was unilaterally injected in the lateral ventricle at three different doses. Results showed a clear dose-response in TPP1 levels in cerebrospinal fluid (CSF) and brain parenchyma.

A significant linear correlation was found in CSF, (r^2=0.91, 0.82, and 0.58, respectively) cerebellum and medulla oblongata. The CSF of CLN2-/- mice injected with the high dose of AAV4CAGhTPP1 (1e11 vg) had an ~187-fold increase in proenzyme expression. The mid dose (5e10 vg) had an ~37-fold increase relative to heterozygous levels. Most importantly, the low dose (1e10 vg) also achieved significantly increased TPP1 proenzyme levels (~2.4-fold increase). In all parenchymal regions tested, TPP1 expression was at least equivalent to heterozygous levels. This indicates that the low dose of 1e10 vg of AAV4CAGhTPP1 is sufficient to provide adequate levels of TPP1 in brain parenchyma. Peripheral tissues including the heart, liver, kidney and spleen were analyzed for the presence of recombinant TPP1. All treatment groups had low but detectable levels of recombinant protein in the spleen, with a positive linear regression among the experimental groups (r^2 = 0.54) reflecting a positive dose-response. Nine mice of the high dose group had detectable recombinant enzyme in the heart (less than 0.06 pmol TPP1/ mg protein). Finally, ependymal expression of hTPP1 improved LINCL symptomatology and neuropathology as well as resting tremor that was completely prevented in the high dose group 5 weeks after injection. Finally, treated $CLN2^{-/-}$ mice showed a signficant survival (15 to 19 weeks of age; study ongoing). In conclusion, hTPP1 expressed from transduced ependyma was widely and stably expressed in the brain, well tolerated and produced a dose-dependent therapeutic benefit in TPP1-deficient mice.

877. Neuropeptide-Based "Drug on Demand" Gene Therapy for Treatment of Focal Epilepsy

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Focal epilepsy represents one of the most common chronic CNS diseases. The high incidence of drug resistance and insufficient responsiveness to surgery pose unmet medical challenges. Patients are at high risk to develop devastating comorbidities. In the quest of novel, disease-modifying treatment strategies neuropeptides represent promising candidates. Our novel approach is based on adeno-associated virus (AAV) vector mediated gene therapy to transduce neuropeptide precursors into the epileptogenic focus in well-accepted mouse and rat models of chronic, drug-resistant temporal lobe epilepsy. The aim is to restore the exhausted neuronal supply of seizure-dampening peptides, allowing their locally restricted release "on demand". Here we provide the "proof of concept" that AAV-mediated dynorphin expression in an established epileptogenic focus leads to complete suppression of seizures over months. Supporting data from peptide action on human hippocampal slice electrophysiology suggest a high translational potential. In mice the debilitating long-term decline of spatial learning and memory is prevented. Moreover, lost learning and memory capabilities are regained in chronic epilepsy. Neuronal dynorphin expression is focally restricted and its release dependent on high-frequency stimulation, as it occurs at the onset of seizures. The novel format of "drug on demand" delivery is viewed as a key to prevent habituation and to minimize the risk of adverse effects, leading to long-term suppression of seizures and of their devastating sequelae.

878. In Vivo Disruption of the Mutant Huntingtin Gene by CRISPR-Cas9 Improves Motor Deficits and Lifespan in a Mouse Model of Huntington's Disease

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Huntington's disease (HD) is a currently incurable neurodegenerative disorder characterized by the loss of medium spiny neurons in the striatum, resulting in cognitive impairment, psychiatric disturbances, and motor decline. HD is caused by an expansion of a CAG trinucleotide repeat in exon 1 of the huntingtin (HTT) gene, which leads to widespread aggregation of the mutant HTT protein and disruption of essential cellular functions. Current therapies for HD only treat the symptoms of disease, and there is thus an urgent need for strategies that can correct the underlying cause of the disorder. The RNA-guided Cas9 endonuclease from CRISPR-Cas9 can be used to treat nervous system disorders caused by autosomal dominant mutations by facilitating the introduction of random base mutations that can disrupt mutant gene function following DNA repair, and we therefore hypothesized that CRISPR-Cas9 could be harnessed to treat HD by disabling the expression of the mutant HTT gene *in vivo*. To test this, we first evaluated the ability of the Cas9 nuclease from *Staphylococcus aureus* to target the mutant HTT gene using an engineered reporter carrying exon 1 of the mutant HTT gene with a pathogenic 96Q repeat fused to a cyan fluorescent protein (CFP) gene, thus linking mutant HTT expression to CFP fluorescence. Following its transient delivery to cells overexpressing this construct, we found that Cas9 and our most efficient sgRNA reduced CFP fluorescence by ~75% ($P < 0.0076$) and mutant HTT protein by ~65% ($P < 0.0001$). We then tested the ability of Cas9 and this sgRNA to disable the mutant HTT gene in the R6/2 mouse model of HD. This transgenic mouse carries the 5' UTR and exon 1 of the human HTT gene with ~120 CAG repeats, and phenocopies aspects of the human disorder, including muscle atrophy, epileptic seizures and the gradual loss of motor function. Following adeno-associated virus (AAV)-mediated delivery of the HTT-targeting CRISPR-encoding AAV vector to the striatum, we observed widespread expression of Cas9 in the targeted area, as well as a significant reduction in mutant HTT protein inclusions in $Cas9^+$ cells ($P < 0.05$). Crucially, R6/2 mice treated by CRISPR-Cas9 had improved motor function, reduced hind limb clasping (a measure of disease progression), and a ~15% increase in lifespan ($P = 0.01$) compared to control animals injected with an AAV vector encoding a non-targeting CRISPR. Thus, our results demonstrate that CRISPR-Cas9 has the potential to provide therapeutic benefit for HD.

880. Intradermal Gene Delivery of the Photosensitive Chloride Channel, iC++, Facilitates Transdermal Light-Mediated Inhibition of Post-Surgical Pain in Rodents

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Most patients who undergo surgical procedures experience acute postoperative pain, but less than half report adequate postoperative pain relief. Current drugs act through analgesic mechanisms not specific to the intrinsic pathways of pain, causing unwanted side-target effects such as nausea, sedation and vertigo, and many become addicted to opioid pain killers after exposure during therapy. Light-activated ion channels and pumps allow specific control of neural function in the central nervous system and have been used to modulate peripheral neurons including nociceptors. Specific inhibition of nociceptors has relieved sensitivity in neuropathic pain states following viral delivery at the sciatic nerve in mice. To test whether pain relief could be extended to acute post-surgical pain, mice were injected intradermally with AAV serotype 6 expressing the novel photosensitive chloride channel, iC++. AAV6 is capable of retrograde uptake by motor neurons in rodents and non-human primates, and we hypothesized that this method could result in specific transduction of cutaneous sensory nerves. As predicted, iC++ expression was observed in nociceptive nerve endings following non-invasive skin injection. Further, expression was sufficient to inhibit pain upon light delivery through skin in the mouse post-surgical pain model. Finally, we compared the AAV6 intradermal and intraneural approaches and found that higher transduction was achieved with nerve delivery, however, more specific targeting was attained with intradermal delivery; resulting in equivalent pain inhibition. Taken together, our results suggest that intradermal delivery of AAV6 expressing iC++ is a simple and attractive clinical approach for treatment of post-surgical pain.

881. GS030-DP, an AAV2.7m8-ChrimsonR-tdTomato Gene Therapy Product for Retinitis Pigmentosa Treatment, is Well Tolerated in Monkeys and *rd1* Mice after Bilateral Intravitreal Administration

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GenSight Biologics, Paris, France

GS030-DP, AAV2.7m8 encoding ChrimsonR, an algal light-sensitive opsin, fused to tdTomato, converts retinal ganglion cells into photoactivatable cells by exposure to 595 nm LED light and is intended to restore vision in retinitis pigmentosa patients. Prior first-in-human trial, a complete GLP safety program was conducted in non-human primates and *rd1* blind mice. Toxicity, biodistribution and immunogenicity were evaluated in normal cynomolgus monkeys. Animals received a single bilateral intravitreal injection of vehicle or GS030-DP (7.21×10^{10} or 7.84×10^{11} vg/eye), and were analyzed at 3 and 6 months post-injection. A dose-dependent ocular inflammation was observed both in anterior segment and vitreous, starting at Day 3, peaking at Day 28 and then improving or resolving over time. In high dose animals, inflammation was associated with a decrease in intraocular pressure. No retinal tissues destruction or reorganization were observed. The inflammation was confirmed histologically at Month 3 and 6 with minimal mononuclear cell infiltration in the retina, vitreous body, ciliary body, anterior or posterior chamber, filtration angle and optic disc at both doses. Anti-AAV neutralizing antibodies (NAbs) in serum were showed to increase from Day 15 to Month 2 and then decreased at Month 3 to plateau until Month 6. Incidence of the response was the same at both doses, but intensity seemed slightly higher in the high dose group suggesting a limited dose-effect. NAbs also developed in a dose-dependent manner in aqueous humor and vitreous, persisting up to Month 6. For both doses, GS030-DP vector DNA mainly distributed in cornea, ciliary body, iris, lens, retina, choroid, spleen, and was rarely quantified in lymph nodes, optic nerve and optic chiasm. Low levels of vector DNA were transiently detected in blood (up to Day 7), and rarely in tears and feces (Day 3). Ocular safety of GS030-DP following exposure to 595 nm LED light was assessed in *rd1* mice, the widely used retinitis pigmentosa model. Mice received vehicle or GS030-DP ($7.84x10^9$ vg/eye) by intravitreal injection in both eyes. After 5 weeks post-injection, mice were anesthetized and exposed to either room light or pulsed (0.82 Hz) 595 nm LED light at $1.4x10^{16}$ or $1.7x10^{17}$ photons/cm^2/s using a purpose-built illumination device. Animals were monitored for 2 weeks following light exposure. No GS030-DP related findings and no LED light related retinal findings were noted at either light intensity. Cataract and corneal findings (edema, vascularization, ulcer) were noted in all groups, and therefore considered secondary to the light exposure procedure. To conclude, GS030-DP was well tolerated in monkeys, inducing a reversible dose-related ocular inflammation. In absence of adverse effects and since the ocular inflammation was less pronounced at the low dose, the NOAEL of a single bilateral intravitreal injection of GS030-DP in non-human primates was considered as $7.21x10^{10}$ vg/eye. In mice, GS030-DP expression combined with 595 nm LED light exposure was not associated with toxicity in retina and optic nerve. These preclinical regulatory studies supported the phase 1/2 clinical trial CTA dossier; trial is now ongoing.

882. Treating Retinitis Pigmentosa with Synthetic and Endogenous Transcriptional Regulators

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The RHODOPSIN (RHO) gene is the most common gene involved in autosomal dominant retinitis pigmentosa (adRP). To silence RHO gain-of-function we designed approaches based on transcriptional regulation. In particular, we show that somatic gene transfer to the RHO expressing cells, rod photoreceptors, of transcriptional repressors based on synthetic DNA-binding proteins with (ZF6-R) without (ZF6-DB) canonical repressor domains or the endogenous transcription factor KLF15 enable targeting a unique human RHO regulatory promoter region and in turn complete RHO silencing. Comparison of specificity by RNAseq showed that ZF6-R and ZF6-DB perturbed

220 and 19 differentially expressed genes (DEGs), respectively in the pig model. KLF15 perturbed 156 DEGs demonstrating high selectivity (on-target) by the three transcriptional regulators. Efficacy assessed by electroretinogram analysis (ERG) in a mouse model of adRP show remarkable preservation of retinal function by the three transcriptional regulators. Collectively, these data support transcriptional silencing as a novel paradigm for the treatment of human inherited disorder by gene therapy.

883. Gene Therapy of AB-Variant GM2 Gangliosidoses in a Mouse Model Using Adeno-Associated Virus Serotype 9

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GM2-ganglioside requires specific synthesis, processing and combination of three gene products, HEXA, HEXB, AND GM2A, for proper hydrolysis within a cell's lysosome. However, mutations in any of these gene products results in a deficiency of Hexosaminidase A (HexA) enzyme activity, which functions in the breakdown of GM2-ganglioside. This can result in a group of neurodegenerative diseases affecting the brain, called GM2 gangliosidoses. There are three forms to this disease: Tay-Sachs disease, Sandhoff's disease or the AB-variant, which is the rarest. AB-variant is characterized by a mutation in the GM2A gene, which encodes the GM2-activator protein that is a required co-factor for the breakdown of GM2 gangliosides by the HexA protein. This mutation results in the build-up of GM2 ganglioside to toxic levels, which results in neuronal destruction. This disease is characterized by rapid neurological decline and death by 4 years old, and there is currently no cure. As AB-variant is a set of monogenic diseases, gene therapy is a plausible and, likely, an effective method of treatment. GM2A deficient mouse models provide an exceptional animal model to study potential therapies, as we share 95-98% of our genomes and acquire many of the same diseases. The effective viral vector, Adeno-associated virus serotype 9 (AAV9), has been proven to be successful in treating other forms of GM2 gangliosidosis in preclinical trials and will be used in combination with the GM2A gene. The aim of this study is to give a one-time treatment of *AAV9.GM2A* viral vector therapy at a dose of 1 x 10^{14} vector genomes per kilogram of mouse. Mice were given treatments at 1-day old via the superficial temporal vein or as adults via tail vein in order to correct this mutation. To analyze the effectiveness of this treatment towards correcting GM2-gangliosidoses AB variant, mice were tested behaviorally to assess coordination, activity and strength. Biochemical and molecular analysis was also performed at 20- and 60-week endpoints through various organs. We hypothesize that an optimized *AAV9.GM2A* treatment will correct the gene deficiency, as well as the observable characteristics of GM2-gangliosidosis AB variant in mice. As expected, preliminary behavioral data does not show any statistical significance, as the phenotypic characteristics in the GM2A deficient mouse model does not develop until after 20 weeks of age. The biochemical data for this short-term cohort showed a decrease in GM2 gangliosides when comparing treated and vehicle mice, however these results were only significant for adult treated mice. Long-term data showed a slight increase in locomotor activity and muscular strength, however not significant, of the treated mice in comparison to the vehicle and treated mice. However, a significant increase in motor coordination was observed in the adult treated mice in comparison to the vehicle treated mice. Further biochemical analysis of the long-term mice will likely show a decrease in the GM2 gangliosides between the vehicle and treated mice, similar to the short-term results. With these positive results, we are hopeful that human clinical gene therapy trials can be implemented in the near future.

884. Genome Editing to Generate a Pig Model of Stargardt Disease Type 1

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Stargardt disease type 1 (STGD1), due to mutations in the *ABCA4* gene, is the most common form of inherited macular degeneration. *Abca4-/-* knockout mice are currently used as animal models of STGD1, however they recapitulate only some of the features of the disease and fail to develop the severe photoreceptor degeneration observed in STGD1 patients. This might be due to the structure of the mouse retina, which largely differs from that of humans. The development of an adequate animal model is thus required to both better understand STGD1 mechanisms as well as testing novel potential therapeutic strategies. The porcine eye shares many similarities with the human eye both in terms of size and structure, including the presence of a large streak-like region in the retina, which is similar to the human fovea and where the cone/rod ratio reaches 1:3/1:5. Additionally, genome editing technologies in pigs are particularly advanced. Thus, we have planned to generate a pig model of STGD1, by exploring either: i. somatic cell nuclear transfer (SCNT) from primary fibroblasts edited using CRISPR/Cas9 technology, to generate *ABCA4* knock-out pigs; or ii. photoreceptor somatic gene transfer of CRISPR/Cas9 with adeno-associated viral (AAV) vectors in adult pigs. Screening of potential CRISPR/Cas9 cutting sites in the pig *ABCA4* gene led to the selection of one gRNA in exon 2 used to edit fibroblasts for SCNT, and 3 gRNAs in exon 2, 5 and 6 for somatic gene editing. Effective editing of the *ABCA4* gene was confirmed in both ABCA4 knock-out (KO) pigs generated through SCNT and in retinal lysates from adult pigs 6 months after subretinal delivery of AAV vectors encoding for Cas9 and the combination of the 3 gRNAs. Notably, *ABCA4* gene modification resulted in undetectable levels of ABCA4 protein production in ABCA4 KO pigs and significant,

although variable, reduction in ABCA4 levels in AAV-Cas9-injected pigs. Further characterization of STGD1 phenotype development in both animal models is in progress. If successful, the generation of an animal model of STGD1 will provide unique tools for both studying the mechanism of STGD1 rod and cone cell death as well as testing new therapies.

885. Enhanced Efficacy of Gene Therapy Treatment for Niemann-Pick C1 Disease Using a Novel Serotype, AAV-PHP.B

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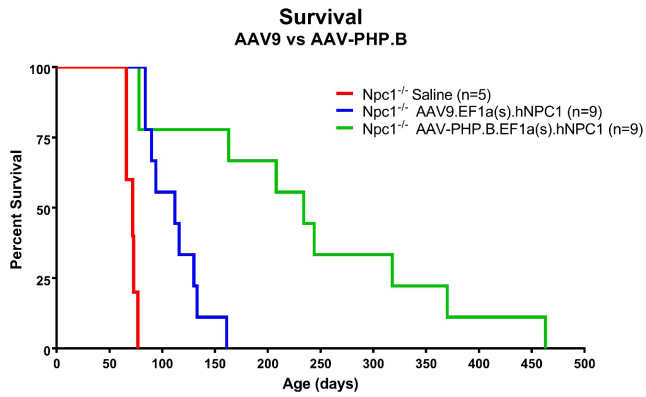
Introduction: Accessing the central nervous system (CNS) continues to present a challenge when developing therapies for the treatment of neurological diseases. Overcoming the barrier of gene transfer to brains of animals and patients from systemic circulation has been difficult. Recent advances using Cre recombination-based adeno-associated virus (AAV) targeted evolution (CREATE) has yielded a promising new serotype, AAV-PHP.B, with greater transduction than AAV9 in the adult mouse CNS after systemic delivery. Here we show systemic delivery of a therapeutic AAV-PHP.B vector outperforms the naturally occurring AAV9 in treatment of a murine model of a rare lysosomal storage disorder, Niemann-Pick C1 (NPC1) disease. Approximately 95% of patients have a mutation in *NPC1* which results in either absence or a significant reduction in functional NPC1, a lysosomal transmembrane protein involved in cholesterol transport. NPC1 pathology involves lysosomal accumulation of unesterified cholesterol and other lipids. Patients typically present with neurological symptoms and visceral complications including hepatosplenomegaly. Disease progression in the null mouse model of NPC1 (*Npc1*$^{-/-}$) is characterized by weight loss, ataxia, and early death. **Results:** We previously reported that systemic delivery of an AAV9 vector expressing the human *NPC1* gene under transcriptional control of a ubiquitous promoter (EF1a) improved lifespan and ameliorated disease phenotype of *Npc1*$^{-/-}$ mice. Using a similar study design, we find that an otherwise identical AAV-PHP.B vector improved lifespan in *Npc1*$^{-/-}$ mice more effectively than an AAV9 vector.



Treatment	Median Survival	Significance (Log-rank test)
Npc1$^{-/-}$ Saline	72 days	
Npc1$^{-/-}$ AAV9	112 days	vs Saline, P < 0.0001
Npc1$^{-/-}$ AAV-PHP.B	234 days	vs Saline, P < 0.0001 vs AAV9, P < 0.005

We also find that AAV-PHP.B-treated *Npc1*$^{-/-}$ mice had a delay in onset of disease progression and motor decline compared to AAV9-treated mice. Vector biodistribution of AAV-PHP.B and AAV9 was determined in liver and cerebrum by droplet digital PCR and exhibited a serotype specific pattern of transduction efficiency, with AAV-PHP.B>AAV9 in brain and AAV9>AAV-PHP.B in liver. Western blots show NPC1 protein expression in liver and cerebrum correlated with vector copy number. Finally, lipidomic analysis of lipid species known to accumulate with NPC disease progression exhibited patterns of tissue specific reduction which correlated to NPC1 transgene expression. **Conclusion:** While these results are among the first to show that AAV-PHP.B, a novel serotype with enhanced CNS tropism, improves disease phenotype in an animal model of neurologic disease, they also suggest that greater CNS transduction for NPC1 disease increases therapeutic efficacy.

886. Understanding and Challenging the Contribution of HSCs to Brain Myeloid Cell Turnover

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We have recently shown that hematopoietic stem and progenitor cells (HSPCs) can contribute to the turnover of brain myeloid cells upon transplantation in recipients pretreated with a proper conditioning regimen. The transplanted HSPCs home to the brain short term after transplantation, engraft locally and give rise to a mature progeny that shares transcriptional, morphologic and functional features with central nervous system (CNS) microglia (Capotondo et al. 2012). The

engrafted myeloid cells can act as vehicles for therapeutics as well as exert critical immuno-modulatory and neuroprotective functions. Based on our findings, we postulated the existence within the HSPCs pool of functional hematopoietic equivalents of microglia progenitors (HE μP) that can replace CNS resident μP in defined experimental conditions. We first demonstrated that HE μP are comprised within long-term hematopoietic stem cells (HSCs)(Capotondo, Milazzo et al., 2017). We then showed that gene marked (GFP^+) HE μP after long-term engraftment in the brain of myeloablated mice retain clonogenic potential, since they are able to give rise to myeloid-like GFP^+ colonies when plated for the colony forming cell (CFC) assay in a semi-solid methylcellulose medium. We then transplanted hematopoietic cells isolated from the brain of primary GFP^+ HSPCs recipient mice into secondary myeloablated recipients to assess whether CNS-engrafted HE μP also retain long-term hematopoietic reconstitution potential. Interestingly, hematopoietic cells retrieved from the brain of primary recipients were able to engraft long-term in hematopoietic tissues and brain of secondary recipients, showing multi-lineage differentiation capability. These results indicate that HE μP retain HSCs functional features upon long-term engraftment into the brain. An integration site analysis on the transplanted GFP-HSPCs and on the tissues isolated from primary donors and secondary recipients is ongoing to strengthen these results with clonal tracking data. Based on these findings, we also hypothesized that CNS-resident μP may be endowed with features reminiscent of HSCs. We are thus experimentally addressing this hypothesis taking advantage of a reporter animal model suitable for murine HSC identification, the Fgd5-Zs green mouse (Gazi et al. 2015) as we already showed that Fgd5 expressing HSCs are enriched in functional HE μP. Overall, the identification and characterization of these cell populations will provide an ideal target for advanced, brain-directed myeloablative conditioning regimens and will allow identifying the ideal population for transplantation purposes for HSPCs transplantation and gene therapy protocols aimed at microglia reconstitution.

887. Surveying the Prevalence of Antibodies that Neutralize Adeno-Associated Virus (AAV) Serotypes 2, 5, 8, and 9 in the Intravitreal Fluids and Sera of Patients with Vitreoretinal Diseases

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Adeno-associated virus (AAV) vectors have been shown to be the most suitable gene delivery tools for the treatment of retinal diseases. However, a significant limiting factor on the efficacy of gene therapy using AAV vectors is humoral anti-AAV antibody mediated immunity, especially pre-existing immunity which results from childhood exposure to wild type AAVs. Nonetheless, there is little information on the pre-existing neutralizing antibodies (NAb) that disrupt AAVs in the human vitreous cavities, which is the main anatomical space used for AAV vectors retinal gene delivery. Here we characterized the prevalence of anti-AAV NAbs against AAV serotypes 2, 5, 8, and 9 by analyzing the in vitro neutralization exerted by intravitreal fluid and sera obtained from thirty patients requiring pars plana vitrectomy (PPV) for treatment of various vitreoretinal diseases. Among the serotypes, NAbs against AAV2 were confirmed most frequent and at highest titers compared with other serotypes in both serum and intravitreal fluid. NAbs against other serotypes were also detected in lower frequencies, and the level of antibody was the lowest in AAV 8. Even though NAbs were present in very high concentrations in sera, NAbs in intravitreal fluid remained low in most cases regardless of AAV serotypes. Relatively higher titers of NAbs were observed in intravitreal fluid from patients with vitreous hemorrhage, of the vitreoretinal diseases including diabetic retinopathy. The results indicate that in most patients, anti-AAV NAbs were found in sera but not in intravitreal fluid, suggesting that the presence of NAbs in sera might not be a limiting factor in using AAV vectors for gene therapy in retinal diseases.

888. Design and Testing of a Codon Optimized RPGR

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Purpose: There are approximately 9000 individuals in the United States alone with *RPGR*-associated retinal disease and 112 new cases per year. As a result, mutations in *RPGR* are the third most common cause of inherited retinal disease and the most common cause of x-linked recessive retinitis pigmentosa (XLRP). The gene encodes two main isoforms, $RPGR^{1\text{-}19}$ and $RPGR^{ORF15}$. The latter is the predominant transcript expressed in mature photoreceptor cells. The two transcripts differ in their terminal exon, and $RPGR^{ORF15}$ contains a low complexity region thought to form triplex DNA. Because mutations in *RPGR* result in a loss of functional protein, gene therapy represents a potential avenue for treatment. Yet, due to the low complexity region, cloning of $RPGR^{ORF15}$ yields an unstable plasmid unsuitable for continual propagation for down stream viral packaging and patient treatment. The purpose of this study was to optimize the $RPGR^{ORF15}$ cDNA and test its expression and function in a relevant cell type. **Methods:** DNA secondary structure predictions were made using Triplex, an R/Bioconductor package. To eliminate candidate triplex forming sequences, base changes were made to the minimum necessary number of wobble positions maintaining the wild-type amino acid sequence. Additionally, a common in-frame deletion found in an unaffected cohort was included. The resulting optimized cDNA ($RGPR^{IOWA}$) and $RPGR^{ORF15}$ were packaged individually in AAV 2/5. For downstream functional testing immortalized RPCs were generated. To accomplish this, iPSCs were made from patient-specific fibroblasts with molecularly confirmed RPGR-associated photoreceptor disease as well as an unaffected line. To generate isogenic controls, disease lines were CRISPR corrected. These iPSCs were then differentiated towards retinal progenitor cells (RPCs) using a stepwise protocol to generate 3D retinal organoids. After 50 days, optic vesicle-like structures were selected manually, dissociated, and plated for 2D expansion. To generate immortalized lines, NRL, CRX,

and hTERT expression cassettes were stably integrated via lentiviral delivery. Disease lines were transduced with *RGPR*^{IOWA} or *RPGR*^{ORF15} viral particles. Cells were fixed and stained for primary cilia related genes as well as RPGR and quantified using confocal microscopy. **Results:** Gene optimization resulted in a more stable vector, which was demonstrated by the number of correct vectors obtained post-transformation. Packaging of both vectors yielded roughly equal titers. iPSC lines were successfully generated and confirmed via the Taqman Scorecard Panel. Both disease lines were CRISPR-corrected and confirmed via Sanger sequencing. Immortalized lines were generated and expressed photoreceptor markers such as RCVRN, NR2E3, Opsins, and RPGR^{ORF15}. As a proof of concept, the unaffected control RPCs contain a primary cilium, which positively stains for typical markers such as RPGR, polyglutamylation, and INPP5E. Moreover, the immortalized RPCS display a transduction efficiency of greater then 20 percent. Both disease lines were successfully transduced with AAV2/5 CMVp *RPGR*^{IOWA} or CMVp *RPGR*^{ORF15}. **Conclusions:** Mutations in RPGR are the third most common cause of inherited photoreceptor disease and a promising candidate for gene therapy. To this end, the removal of potential triplex forming sequences yielded a stable vector suitable for large-scale virus production. While animals are ideal for safety testing, iPSC technology allows for testing the function of treatments in a relevant human cell type.

889. Overexpression of NAMPT in Astrocytes by AAV is Brain Protective in Focal Ischemic Stroke

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We previously demonstrated that nicotinamide phosphoribosyltransferase (NAMPT), a rate limiting enzyme in the salvage pathway of NAD^+ biosynthesis in mammals, is primarily expressed in neurons under normal conditions and is brain protective after ischemic stroke in a mouse model of photothrombosis (PT). Here we showed that NAMPT is largely upregulated in reactive astrocytes in the peri-infarct region (PIR) after PT. In addition, the upregulation of astrocytic NAMPT is time and spatial dependent in peri-infarct region. To test whether astrocytic NAMPT is brain protective in ischemic stroke, we generated serotype 2/5 constructed AAV vectors encoding NAMPT with astrocyte specific promotor gfaABC1D, i.e., AAV2/5-gfaABC1D-NAMPT. We injected the AAV vectors in the cortex two weeks before PT and sacrificed 7 and 14 days after PT. Our results show that NAMPT is selectively expressed in astrocytes after AAV transduction and the mice injected with AAV exhibit smaller infarct volume. Moreover, the astrocytes overexpressed with NAMPT exhibit higher percentage of SOX2 expression, a neuronal stem cell marker. Our study thus suggests overexpression of NAMPT in astrocytes is brain protective and contributes to reactive gliosis and cell proliferation in focal ischemic stroke.

890. BBB Disruption Enhances the Efficacy of Systemic AAV9 Gene Delivery in GM1 Gangliosidosis Mice

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Neuronal transduction by AAV vectors is highly efficient upon local delivery. However, after systemic delivery, central nervous system (CNS) transduction requires crossing the blood-brain barrier (BBB). In adults, the BBB is formed by highly regulated tight junctions in the brain endothelia with very low permeability to macromolecules. One approach to enhance the efficiency of AAV9 CNS gene transfer is to temporarily disrupt the BBB, which has been successfully achieved by focused ultrasound. Here we tested a new approach to increase the efficiency of systemic AAV9 CNS gene delivery through the co-administration of vector with a previously described BBB disruptive agent. Initial studies using young adult Balb/cJ and C57Bl/6J mice were performed by co-injecting in the tail vein 5E11 vg AAV9 vector encoding Firefly luciferase (FLuc) with different doses of the BBB-active agent. At 3-weeks post-injection, FLuc activity was measured in CNS and liver and we observed an increase of relative enzyme activity of ~600 fold in brain but no changes in liver. This enhancement in FLuc activity in the brain was also observed for AAVrh10. In order to test the therapeutic potential of this approach, we conducted short-term biochemical efficacy studies in GM1-gangliosidosis mice treated systemically with AAV9-mβgal vector in the presence or absence of BBB active agent. GM1-gangliosidosis is a lysosomal storage disease caused by mutations in the *GLB1* gene that affect the activity of lysosomal acid beta-galactosidase (βgal), and result in progressive accumulation of GM1-ganglioside in the CNS and neurodegeneration. The *Glb1*$^{-/-}$ mouse model has no residual βgal activity and stores massive amounts of GM1-ganglioside in the CNS. Previous studies have shown that systemic administration of 3E11 vg of AAV9 encoding mouse βgal (mβgal) significantly reduce the GM1-ganglioside content in CNS but was insufficient to restore it to normal levels. Here *Glb1* KO mice were treated at 6 weeks of age with 1E11 vg or 3E11 vg of AAV9-mβgal with or without BBB-active agent. At 4 weeks post-injection there was a significant increase in βgal activity in the brain of all groups, but animals co-treated with AAV9 and the BBB-active agent showed βgal levels ~3-fold higher than age matched normal controls. Histological analysis of storage in the brain using Filipin staining of tissue sections showed that co-administration of 1E11 vg AAV9-mβgal and BBB-active agent was effective in eliminating GM1-ganglioside storage in the brain. The combination of systemic AAV9 CNS gene delivery with an agent that temporarily disrupts the BBB in a global manner is a powerful approach to potentiate the efficacy of this promising gene therapy approach for GM1 gangliosidosis and possibly other neurological disease.

891. TALEN and CRISPR Gene-Editing for Treatment of Machado-Joseph Disease

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Machado-Joseph disease (MJD) is a fatal and untreatable, dominant neurodegenerative disorder. It is caused by an unstable expansion of a CAG tract in the coding region of the *ATXN3* gene, resulting into a polyglutamine repeat expansion (1). This confers a toxic gain-of-function to the resultant ataxin-3, leading to the formation of neuronal intranuclear inclusions and to cell death (2). Specific gene correction or inactivation can be achieved through engineered nucleases, such as TALENs and CRISPR/Cas systems (3). Upon the introduction of targeted double strand breaks, genome editing is attained through the activation of endogenous machinery, facilitating the generation of knock-out and knock-in models (4). In order to suppress *ATXN3* gene expression through the insertion of a loss-of-function mutation, a panel of sequences (TALENs and CRISPR), directed against an early exon of the human gene, were designed and constructed. Functional characterization was performed in HEK 293T cells, through the surveyor mutation detection assay. One sequence of each system was selected and intra-cranially delivered to an *in vivo* lentiviral mouse model of MJD, using adeno-associated viral particles. The neuropathological markers were assessed 4 weeks after surgery. Surveyor mutation detection assay revealed the editing capability of our customized nucleases both in HEK 293T cells, and in striatal samples of the mouse model. We observed a reduction in the levels of wild-type ataxin-3 in human cells and a drastic reduction of the mutant protein in the animal model, in a dose-dependent manner. Immunohistochemical analysis of mouse brain sections revealed the same tendency for the reduction of aggregates in the striatum. Genome editing technologies based on programmable nucleases opened up the possibility of treating diseases that are caused by gene mutations at their source. Accordingly, our results suggest that both TALEN and CRISPR systems are able to efficiently target and modify the *ATXN3* gene, leading to the insertion of a loss-of-function mutation, and consequently to its knock-out. References : 1 - Kawaguchi, Y *et al*. Nat Genet. 1994; 8(3): 221-8; 2 - Bettencourt, C *et al*. Orphanet J Rare Dis. 2011; 6:35; 3 - Cox DB *et al*. Nat Med. 2015; 21(2):121-31; 4 - Gaj T *et al*. Trends Biotechnol. 2013; 31(7):397-405.

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892. Towards a Gene Therapy for Autosomal Dominant Retinitis Pigmentosa Caused by Mutations in Rhodopsin

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The retina is a highly-specialized organ that houses light-sensitive photoreceptor cells, which initiate the first steps in our vision, as a well as a network of neurons that relay visual information to the brain. A cast of supporting cells, including microglia, Müller glia, and the retinal pigment epithelium, ensure the proper functioning and health of the photoreceptors and neurons of the retina. The high level of specialization of the retina makes it particularly susceptible to genetic mutations. In fact, over 250 genes, when mutated, can result in degeneration of the retina and impairment of vision. The most common form of inherited retinal degeneration is Retinitis Pigmentosa (RP), which afflicts 1.5 million individuals worldwide. In RP, an inciting mutation directly and negatively affects the survival of rod photoreceptor cells, resulting in constriction of the peripheral visual field as well as loss of night vision. With substantial loss of rods in RP, death of cone photoreceptors occurs through a poorly understood mechanism, which can leave a patient completely blind. At present, RP remains incurable. The major goal of our research program is to develop new knowledge regarding gene therapies, utilizing recombinant adeno-associated virus (rAAV) as a vector for gene delivery, to slow vision loss in RP. The major barrier in generating a successful therapy arises due to the genetic heterogeneity of RP, as mutations in over 60 genes can lead to the stereotypical photoreceptor loss seen in RP. Tailoring a treatment to each mutation would be laborious and expensive. The aim of this abstract is to discuss our laboratory's approaches to designing efficacious rAAVs that are also applicable to many of the genetic forms of RP. Our first strategy involves targeting a common subtype of RP caused by autosomal dominant mutations in the rod-specific protein, rhodopsin (RHO). Here, co-delivery of a short-hairpin RNA (shRNA) to remove endogenous RHO as well as a shRNA-resistant RHO cDNA for gene replacement with a rAAV vector should preserve photoreceptors and vision in RHO-mediated RP. The current paradigm in the field of RP suggests that cone death occurs through a common pathway, regardless of the inciting mutation, which involves noxious inflammatory signaling arising from the supporting glial cells of the retina. To this end, our second approach seeks to protect cones in RP by delivery of an anti-inflammatory pox virus protein (M013) or a nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activating peptide with a rAAV to abrogate damaging inflammation. The rAAV gene therapy strategies discussed here should be applicable to other disease processes, inside or outside the retina, with an autosomal dominant origin and/or an inflammatory component.

893. Characterization of Microglia Responsive Promoters for Optimized Gene Therapy of Neurodegenerative Diseases

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Many neurodegenerative diseases could be susceptible to benefit from hematopoietic stem cell (HSC) gene therapy (GT) and their ability to colonize the central nervous system (CNS) with engineered myeloid cells. However, adaptation of current protocols would be relevant to address disease and nervous system peculiarities and to increase the likelihood of benefit, including choice of ideal vector cassettes, conditioning regimes and infusion routes. In this context of our efforts to enhance the benefit of HSC GT for CNS conditions by engineering specific vectors, we introduced in our lentiviral vectors (LVs) and characterized the promoter of a well-known microglia stress-induced marker as a tool for a more sensible and specific gene delivery in the environment of neurodegenerative diseases cursing inflammation. The 18-kilobase translocator protein (TSPO) is a nuclear encoded mitochondrial protein whose induction has been extensively characterized in neurodegenerative conditions such as Parkinson or Alzheimer's disease. We demonstrated that lentiviral vectors harboring GFP under the control of a full lenght (2.7 kb) mouse *Tspo* promoter were able to drive GFP expression and to reproduce physiological TSPO induction upon LPS treatment in a microglia cell line. Then, we investigated if shorter versions of the promoter, more suitable for vectorization, could be used without loss of the *Tspo* physiological modulation. We cloned a range of *Tspo* regulatory sequences from 300 bp to 2700 bp. Next, we transduced immortalized cell lines of fibroblast and microglia origin, and then selected stable vector harboring clones. Our results showed that as little as 320 bp of the *Tspo* regulatory sequence are sufficient to drive the expression of GFP. Interestingly, the strength of the expression driven could be correlated with the length of the *Tspo* promoter. Furthermore, we could demonstrate no effect of LPS on the reporter expression of transduced fibroblast, while, all *Tspo* promoters responded to LPS stimulation with an increase of GFP expression in microglia cells. These results strongly encourage the validation of *Tspo* regulatory elements for *in vivo* HSC gene therapy and set the foundations for a more specific generation of microglia targeting lentiviral vectors to treat neurodegenerative diseases.

894. iPSC-Derived Neurospheroids Recapitulate Development and Pathological Signatures of Human Brain Microenvironment

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Brain microenvironment plays an important role in neurodevelopment and function. Disruption of its homeostasis is often related to pathological conditions, as in mucopolysaccharidosis type VII (MPS VII). MPS VII is a neuronopathic rare lysosomal storage disease (LSD) caused by deficient β-glucuronidase activity, leading to an abnormal accumulation of glycosaminoglycans (GAGs). We hypothesized that 3D differentiation of human neural stem cells (hNSC) neurospheroids in perfusion stirred-tank bioreactors could sustain microenvironment remodeling, recapitulating key cell-ECM interactions. Differentiation of hNSC derived from induced pluripotent stem cells (hiPSC-NSC), both from healthy donors and a MPS VII patient, were shown to recapitulate neurogenic developmental pathways, generating tissue-like 3D structures with neuronal, astroglial and oligodendroglial cells. Changes in neural microenvironment during differentiation, namely at cell membrane and ECM composition, were addressed using quantitative transcriptomic (NGS) and proteomic data (SWATH-MS). Data revealed a significant enrichment in structural proteoglycans, such as neurocan, versican, brevican and tenascin C, along with downregulation of basement membrane constituents (e.g., laminins, collagens and fibrillins). In MPS VII cells, main disease hallmarks were recapitulated, e.g. the accumulation of GAGs. MPS VII neurospheroids showed an upregulation of the glial cell marker GFAP, which was associated with astrocyte reactivity, and downregulation of GABAergic neuron markers. Notably, calcium imaging analysis in MPS VII neurospheroids showed reduced neuronal activity and disturbances in network functional connectivity. These data provide insight into the interplay between reduced β-gluc activity, GAG accumulation, alterations in the neural network, and its impact on MPS VII-associated cognitive defects. In summary, we demonstrated that neural cellular and extracellular developmental features are recapitulated in hiPSC-NSC-derived neurospheroids and MPS VII-associated perturbations were detected through hiPSC-NSC neuronal differentiation. The 3D neurospheroids are a valuable *in vitro* model to address molecular and cellular processes associated with neurological disorders that affect the microenvironment homeostasis, as MPS VII and other LSD.

895. Evaluation of Therapeutic Potential of Human *USH2A* Gene Lacking Exon 13 (*USH2A*-ΔEx13) for Restoring Ciliogenesis

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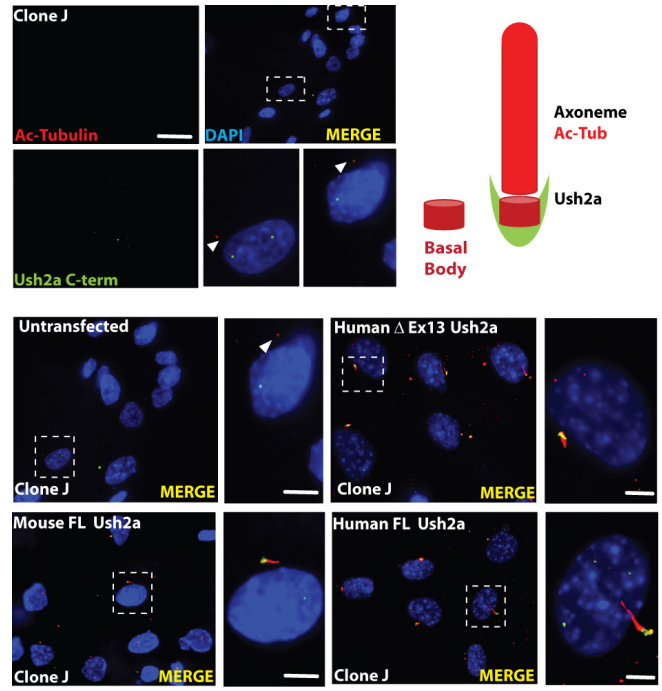
Rationale: Mutations in *USH2A* are the most common cause of Usher syndrome type II, involving visual and hearing loss. It is also the most common cause of inherited retinal degenerations (IRDs) overall. Approximately 30% of USH2A cases are caused by the c.2299delG, p.Glu767fs mutation in exon 13 of the *USH2A* gene. Development of gene therapy for the *USH2A* form of arRP has been challenging, because the full length CDS of *USH2A* (15.6kb) far exceeds the packaging capacity of commonly used AAV viral delivery vectors for gene augmentation therapy. The *USH2A* gene encodes a transmembrane protein anchoring in the photoreceptor plasma membrane. There are multiple repetitive domains, including 10 laminin EGF-like domains and 35 fibronectin type 3 domains at its N terminus of USH2A. The frame-shift mutation c.2299delG, p.Glu767fs is located in exon 13 in LE domain 5. Skipping of this exon 13 (642bp) will result in an in-frame abbreviated transcript USH2A-ΔEx13. In this study, we propose to examine if the product of this abbreviated USH2A-ΔEx13 will retain partial or complete biologic function of USH2A protein. **Results:** We chose OC-k1 cells as our modeling system for this study. OC-k1 cells are derived from mouse cochlea and express Ush2a protein and its interacting proteins at the base of the cilia under serum deprived conditions. We first created an *Ush2a* null cell model in OC-k1 cells using CRISPR/Cas9 technology. Guide RNAs were designed to target the exon 5 of mouse Ush2a gene. Depleting of the all three Ush2a alleles in the OC-k1 cells were confirmed by NGS analysis. We demonstrate that ciliogenesis was hampered in the *Ush2a* null OC-k1 cells, emphasizing the important role of Ush2a protein in ciliogenesis. Moreover, our transient transfection experiments unequivocally demonstrate that the ciliogenesis in these cells can be restored using DNA plasmids expressing mouse full length (mUsh2a-FL), human full length (hUSH2A-FL) and human USH2A lacking exon 13(USH2A-ΔEx13). **Therapeutic Potential:** Our experiments clearly demonstrate that the human Ush2a protein that lacks Exon 13 (which is a hotspot for frequent deleterious mutations that leads to Usher syndrome type II) has the ability to restore cilia formation in null OC-k1 cells to a similar extent seen with full length human and mouse USH2A proteins. These studies support the design of CRISPR/Cas9 exon13 editing strategy for restoring photoreceptor and auditory function in patients with Usher II syndrome.



896. Intravitreal Delivery of the AAV2quad+T-V/smCBA-sGFP-TatNrf2mer in Two Models of RPE Oxidative Damage

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The retina is one of the tissues with the highest oxygen demand in the body. Its high metabolic rate makes it vulnerable to oxidative damage which is known to occur in different retinal degenerative diseases. In the case of age-related macular degeneration (AMD), it is believe that the retinal pigmented epithilium (RPE) is affected by the oxygen radicals generated in damaged mitochondria. We have developed an AAV vector that allows the expression of a secretable and cell-penetrating peptide derived from the Nrf2 transcription factor (TatNrf2mer). This TatNrf2mer is capable of interacting with the Keap1 protein which normally binds to Nrf2 and induces its degradation. The result is the activation of endogenous Nrf2 and the expression of antioxidant enzymes such as HO-1. Herein we show that intravitreal delivery of the AAV2quad+T-V/smCBA-sGFP-TatNrf2mer vector can partially protect the retina from acute oxidative damage by the upregulation of antioxidant genes. This vector is currently being tested in transgenic mouse model of RPE-oxidative damage which recapitulates certain features of geographic atrophy, an advanced form of AMD.

897. Developing a CRISPR *In Vivo* Platform to Expedite Drug Target Validation

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CRISPR-Cas9 is a genome editing technology with a broad range of applications from therapeutics to functional annotation of gene elements. A Cas9 knock-in mouse has been developed, and will facilitate application of CRISPR-Cas9 *in vivo*. Our ultimate goal is to utilize the Cas9 knock-in mice to establish a platform that will expedite *in vivo* studies of drug target validation for neurodegenerative diseases. Here, we characterized expression of Cas9 proteins in various subregions and cell types in central nervous system (CNS) by immunohistochemistry. In addition, we compared a number of *in vitro* and cell-based assays for their reliability in measuring the efficiency of guide RNAs. Lastly, we delivered guide RNAs into CNS of the Cas9 knock-in mice by stereotactic injection of AAV, and identified key parameters that may enhance efficiency of CRISPR-mediated gene disruption *in vivo*. In future studies, the Cas9 knock-in mice will be crossed to mouse models of CNS disorders. Following CRISPR-mediated disruption of disease-associated genes, alleviation of pathology and behavior deficits in disease mouse models will be assessed in a battery of histological, eletrophysiological and behavior tests.

898. Gene Editing of C9FTD/ALS Expanded $(GGGGCC)_n$ Repeat Using CRISPR/Cas9 System in Mice

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ALS is a progressive neurodegenerative disease that affects motor neurons in patient's brain and spinal cord. ALS prognosis is very poor with most patients dying within 5 years of diagnosis. A major breakthrough for ALS came in 2011 when a hexanucleotide repeat expansion in the non-coding region of a gene called *C9ORF72* was reported to be the most common cause of both inherited (40%) and sporadic (5-6%) ALS. While healthy individuals have less than 30 repeats, patients' expanded repeats can reach up to 1600. This disease is referred to as C9FTD/ALS because it also causes inherited frontotemporal dementia (FTD). C9FTD/ALS is characterized by classic ALS presentation and prognosis but has clinical overlap with FTD. C9FTD/ALS patients can experience dementia with deterioration in behavior, personality, and language in addition to loss of motor function. Both ALS and FTD are aggressive diseases with no treatments to significantly slow disease or extend life expectancy. Nevertheless, three major hypotheses have emerged to explain this expansion's pathogenicity: 1)Haploinsufficiency due to reduced C9ORF72 transcript levels and thus its gene products, 2)nuclear RNA foci formation which leads to RNA and RNA binding proteins sequestration, and 3)toxic dipeptide proteins produced through repeat-associated non ATG (RAN) translation. Despite the explosion in the number of papers published about C9ORF72 in recent years, the mechanism behind this $(GGGGCC)_n$ repeat expansion toxicity has not been identified. In fact, mutations in more than 25 genes have been linked to ALS since the discovery of SOD1 in 1993, yet we still have a limited mechanistic understanding and no cure for ALS caused by these genes.Despite the lack of clear pathophysiology of these genes, it remains accepted that these genes and their products are the source of a toxic-gain-of-function mechanism. Thereby the field of gene therapy and gene editing poses suitable treatment modalities for these disorders as they would modulate this toxicity at the genetic level. An attractive way to treat C9FTD/ALS is to physically erase the repeat expansions from the C9ORF72 genomic locus and restore the gene to a normal or healthy state. Until recently, such a feat would have seemed insurmountable. However, with the discovery of CRISPR/Cas9-guided genome editing and human clinical trials with this technology already underway, such an approach is now within reach. CRISPR/Cas9 is a nuclease that can make double-strand breaks in genomic DNA. It is guided to the target sequence by an associated guide RNA (gRNA) with ~20 nts of complementarity. Current gene therapy approaches being developed with CRISPR/Cas9 involve delivery of the Cas9 enzyme with a gRNA via AAV vectors. This removal may alleviate the cause of ALS in patients with C9ORF72 specific mutations without the need to solve the underlying pathology of these repeats. Thus, gRNAs were designed to target the repeat region and were first tested in Hek-293 cells to determine the most efficient gRNAs to be packaged in AAV9. Our group has previously generated a BAC111 mouse model expressing human C9Orf72 with an expanded $(GGGGCC)_{300\text{-}500}$ repeats which we used to test gene editing both *in vitro* and *in vivo*. Here we show that we can gene edit C9ORf72 by deleting the expanded repeat in primary cortical neurons in culture and in mice tissue through various delivery routes of CRISPR/Cas9 system. This editing alleviates some of the hallmarks of C9FTD/ALS such as foci formation and dipeptide production making this technology promising for future clinical applications.

899. Developing Treatments for Dominant Retinal Dystrophies Using Patient-Specific Induced Pluripotent Stem Cells

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Inherited retinal disease is a major cause of blindness worldwide. A recent survey of one thousand consecutive families with clinical diagnoses of inherited retinal disease determined the molecular etiology of the disease in 76% of these families. Of these, approximately 12% carried disease-causing, dominantly inherited variants, of which 36.5% lay within the gene Rhodopsin (*RHO*). The most common disease-causing mutation in patients with *RHO*-associated retinitis pigmentosa (RP) is a dominant gain-of-function missense variant at amino acid residue 23 (Pro23His). The histidine substitution creates a mis-folded protein, formation of protein aggregates within the cell, ER stress and, eventually, photoreceptor cell death. Treatment for this disease and other dominantly inherited retinal dystrophies will require knockdown or ablation of mutant allele expression. In this study we address this challenge by employing CRISPR-Cas9-mediated

transcriptional repression. We aim to mitigate the pathophysiology of the dominant Pro23His mutation, the most common disease-causing rhodopsin (*RHO*) mutation in patients with *RHO*-associated retinitis pigmentosa (RP) in patient-derived retinal progenitor cells. We created CRISPR-Cas9 transcriptional knockdown reagents using catalytically inactive *S. pyogenes* Cas9 (dCas9) fused to the Krüppel associated box (KRAB) transcriptional repressor domain and single guide RNAs (sgRNAs) targeted to the promoter region of the human *RHO* gene. We evaluated six guides targeted to transcriptional regulatory elements in the promoter region of *RHO.* Using a reporter construct carrying GFP cloned downstream of the *RHO* promoter fragment (nucleotides -1403 to +73), we assayed GFP knockdown in cells treated with our *RHO* promoter-targeted CRISPR-dCas9 reagents. Quantitative RT-PCR assays in cells treated with CRISPR-dCas9 and reporter plasmids demonstrated 78%-84% reduction in GFP expression when compared to control cells treated with reporter plasmid only. The guide with the greatest knockdown in this assay will be used to assess reduction of ER stress associated with the Pro23His mutation in patient-derived retinal progenitor cells. This work may provide a paradigm from which to develop CRISPR-dCas9 therapies to treat other dominantly inherited retinal dystrophies.

900. Prevalence of Aromatic L-Amino Acid Decarboxylase Deficiency in At-Risk Populations

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Background: Aromatic L-amino acid decarboxylase (AADC) deficiency is an ultra-rare, autosomal recessive metabolic disorder characterized by pathogenic mutations in the *DDC* gene, resulting in the loss of AADC enzyme activity. As AADC is required for dopamine and serotonin synthesis, AADC deficiency leads to severe neurologic impairments, including developmental delay with hypotonia, oculogyric crisis, and autonomic dysfunction. Current treatment options are limited, and gene therapy represents a promising new therapeutic strategy. To date, there are approximately 120 known patients diagnosed with AADC deficiency in the literature, with 50 reported of Asian ethnicity and the remaining of non-Asian ethnicity; thus, the disorder is not exclusive to Asian populations and has been documented globally, including in the US and Europe. However, the prevalence outside of Taiwan has not been established. Therefore, a retrospective study was conducted to determine a prevalence estimate within an at-risk population for AADC deficiency. **Methods**: 18,647 requests were received for cerebrospinal fluid (CSF) neurotransmitter metabolite analysis from patients with neurological deficits of unknown origin at Medical Neurogenetics (MNG) Laboratories in the US between the beginning of 2008 and the end of 2016. Patients with CSF neurotransmitter metabolite profiles consistent with AADC deficiency, together with studies examining AADC plasma enzyme assay and Sanger sequencing of the *DDC* gene, were used to estimate prevalence of AADC deficiency. **Results:** 36 new cases of AADC deficiency were identified. Of the 36, 22 were initially identified from CSF analysis, 9 from plasma AADC enzyme assay, and 5 following sequencing of the *DDC* gene. Additional follow-up confirmatory *DDC* sequencing was performed on 17 patients in whom diagnosis was initially made from either CSF or plasma enzyme activity. 25 different pathogenic variants were identified. 15 had not previously been published in the literature or in CLINVAR. Of 44 alleles from 22 patients in whom sequencing was performed, only 8 alleles contained the c.714+4A>T (IVS6+4A>T) pathogenic variant most commonly found in Taiwanese patients. In previous studies, screening for AADC deficiency in an at-risk population in Asia identified a prevalence of 0.25%,[1] and newborn screening in Taiwan established the overall incidence at 1:32,000 births.[2] In the present study of an at-risk population, an estimated prevalence frequency of 0.193% was calculated. The lower frequency in this study may be explained by the presence of a founder variant in the Taiwanese population. Assuming that prevalence frequencies in at-risk populations are indicative of newborn incidence rates, we used the Asian newborn incidence frequency and relative difference in prevalence frequency between the present and Asian study to extrapolate that newborn incidence in an at-risk population is approximately 1:41,000 births. If the patients diagnosed initially by sequencing or plasma enzyme assay are not included with those diagnosed by CSF testing, the prevalence estimate is 0.117%, and the newborn incidence estimate is approximately 1:68,000 births. **Conclusions:** The estimated prevalence from an at-risk population suggests that AADC deficiency is not uncommon, especially in an at-risk population tested for possible biogenic amine neurotransmitter disorders. This raises the possibility that there are patients with AADC deficiency who have not been identified and treated. Timely identification of patients with AADC deficiency is critical to providing appropriate management, such as the developing novel gene therapy approaches.

References:

1. Lee HC, Lai CK, Yau KC, et al. *Clin Chim Acta*. 2012;413(1-2): 126-130.
2. Chien YH, Chen PW, Lee NC, et al. *Mol Genet Metab.* 2016;118(4): 259-263.

901. Preclinical Study of Novel Tetracyclic Small Molecule, NSC745887, for Brain Cancer

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The development of a new generation of therapeutic drugs for malignant gliomais an active area in both chemistry and cancer research. Previously, we reported that the small molecule naphtho[2,3-f]quinoxaline-7,12-dione (NSC745887) traps DNA-topoisomerase cleavage complexes, and effectively inhibits the proliferation of a variety of cancers.In this study, we further explored the activity of NSC745887using U118MG and U87MG human glioblastoma cells and elucidated the related mechanisms of action. NSC745887 induced DNA fragmentation, cell cycle arrest, mitochondrial membrane potential change and the apoptosis-mediated signaling pathway.Moreover, NSC745887 clearly induced accumulation of glioblastoma (GBM) cells in the subG1 and G2/M phases in a dose- and time-dependent manner.Screening DNA damage in GBM cells by levels of γH2AX and DNA fragmentation indicated that treatment with NSC745887 attenuated the expression of decoy receptor 3 (DcR3) and the mitochondrial membrane signaling cascade in GBM cells.This observation was further confirmed by Western blotting. Mechanistic investigation demonstrated that

NSC745887 not only inhibited the caspase-8 mediated downstream signaling pathways but also suppressed poly (ADP-ribose) polymerase (PARP) and reduced of fluorine-18 fluorodeoxyglucose (^{18}F-FDG) specific uptake in GBM cells.These findings establish lead compound potency and provide further insights into the mechanism of action of this new small-molecule anti-glioblastoma agent NSC745887,which we consider to be suitable for further development for the treatment of glioblastoma.

902. AAV Gene Therapy in a Canine Model of MPS1 Prevents and Reverses Corneal Blindness

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Mucopolysaccharidosis1 (MPS1) is an autosomal recessive lysosomal storage disease resulting in severe phenotypes including neurocognitive defects, cardiac disease, and visual impairment due to corneal clouding. MPS1 is caused by mutations in the gene encoding alpha-L-iduronidase (IDUA), a ubiquitous enzyme that catalyzes the hydrolysis of glycosaminoglycans. Hematopoietic stem cell transplantation (HSCT) has proven to be successful at providing IDUA replacement therapy resulting in increased mental development and prolonged lifespan, sometimes by decades. However, HSCT fails to prevent the MPS1-associated vision loss, which combined with ensuing deafness, results in a vertigo-like state severely compromising the patient's quality of life. To generate a therapy for MPS1 corneal clouding, an AAV-IDUA gene addition strategy was investigated in the MPS1 canine model. A single intrastromal vector administration was well tolerated when administered to pre- and post-symptomatic corneas. As early as 1 wk post-injections, corneal clearing was evident in post-symptomatic MPS1 canines which progressed to complete clarity throughout the duration of the experiment, despite an inconsistent and transient corneal edema. The pre-symptomatic canine did not develop corneal clouding in the AAV-IDUA injected while the storage disease progressed in the AAV control contralateral cornea. Post-mortem histological analyses demonstrate significant correction of multiple disease indicators that directly correlated to IDUA persistence. Vector biodistribution was contained to the eye, however, the highest administered dose resulted genome detection in the liver and an antibody response to the AAV capsid. The collective results demonstrated that a single injection of AAV-IDUA gene therapy is safe and effective for treating MPS1 corneal clouding in a canine model. Although initially envisioned for MPS1 patients that have received HSCT, this strategy is applicable to all MPS1 patients and slight permutations could extend its relevance to other lysosomal storage diseases that manifest similar ocular symptoms.

Pharmacology/Toxicology Studies or Assay Development

903. Dose-Limiting Acute Toxicity Events in Nonhuman Primates after High-Dose Systemic AAVs

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Neurotropic adeno-associated virus (AAV) serotypes, such as AAV9, have been demonstrated to transduce spinal alpha motor neurons when administered intravenously at high doses to newborn and juvenile animals. This observation led to the recent successful application of intravenous AAV9 delivery for infants with spinal muscular atrophy type I. In further exploring the potential of high dose systemic AAV to treat neurological diseases, we evaluated the consequences of delivering high doses of AAV9 variant vectors into the systemic circulation. In the first program, we treated three juvenile NHPs (13-14 months) with 2×10^{14} genome copies (GC)/kg intravenously of a clade F AAV vector that is similar to AAV9 carrying a human *SMN* transgene. Administration resulted in widespread transduction of spinal motor neurons; however, severe toxicity occurred in treated NHPs. All three NHPs exhibited marked transaminase elevations within the first week after injection, and one animal was euthanized 5 days post injection with clinical and histologic findings associated with liver failure, intravascular coagulation, bleeding and shock. In the second program, we treated two adult NHPs (4 years) with 7.5×10^{13} GC/kg of either an AAV9 or an AAV9-PHP.B (AAV9-based engineered capsid) carrying a GFP transgene. Both animals developed moderate (AAV9) to severe (PHP.B) elevation of transaminases on Day 3 post injection, with icteric serum and hyperbilirubinemia present in the PHP.B animal only. The AAV9 animal remained clinically unremarkable throughout the study duration, whereas the animal that received PHP.B developed leukopenia, thrombocytopenia, anemia, and a progressively worsening hemorrhagic diathesis with severe petechial to ecchymotic cutaneous hemorrhage requiring humane euthanasia on Day 5. The histologic findings consisted of acute hemorrhage within the diaphragm, heart, and subcutis and marked multifocal single hepatocellular necrosis and degeneration with marked cholestasis. Clinical pathology, gross findings, and histology were compatible with acute liver injury with endothelial cells and/or platelet activation, leading to consumption thrombocytopenia and secondary hemorrhages. While the events reported here have different clinical presentations and possibly different pathogenesis, their acute timing (within days after injection) suggests a direct AAV-mediated toxicity when administered intravenously at high doses. Additional NHP studies are needed to determine the precise pathological and molecular mechanisms involved as well as the potential implication for clinical trials using comparable doses of systemic vectors.

904. VIVO: A Validated and Sensitive Genome-wide Strategy for Identification of CRISPR-Cas Nuclease Off-targets In Vivo

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One major concern with use of site-specific nuclease-mediated gene editing for clinical applications is off-target activity, which can cause genomic instability and disruption of normal gene function. For *ex vivo* therapeutic applications, previously published cell-based genome-wide methods provide potentially useful strategies to identify and quantify off-target mutation sites. However, to our knowledge, a well-validated method that can identify off-targets *in vivo* has not been described to date. Here we describe the validation of VIVO (Verification of In Vivo Off-targets), a highly sensitive and generalizable strategy that can robustly identify genome-wide CRISPR-Cas nuclease off-target effects *in vivo*. VIVO consists of two steps: (1) an initial *in vitro* "discovery" step in which potential off-target cleavage sites are identified with a nuclease of interest on purified genomic DNA using the recently described CIRCLE-seq method; and (2) a subsequent *in vivo* "confirmation" step in which potential genomic off-target sites identified by CIRCLE-seq are examined for evidence of indel mutations in target cells or tissues *in vivo* that have been treated with the nuclease. To test the efficacy of VIVO, we constructed a *Streptococcus pyogenes* (SpCas9) guide RNA (gRNA) intentionally designed to have a high likelihood of inducing multiple off-target mutations in the mouse genome. This promiscuous gRNA (gP) targets a sequence within the coding sequence of exon 1 in the mouse *Pcsk9* gene and was chosen because a large number of closely related sites are also present in the mouse genome. We envisioned that this gRNA could then be efficiently delivered and expressed together with SpCas9 to mouse liver using adenoviral vectors to assess off-target mutations *in vivo*. We used the gP gRNA to target *Pcsk9* with SpCas9, delivering these components intravenously into mice using adenoviral vectors. We achieved high efficiency *in vivo* gene editing of *Pcsk9*, and inducing decreases in plasma cholesterol and Pcsk9 protein levels. *In vitro* CIRCLE-seq analysis performed with gP gRNA and SpCas9 on genomic DNA from mouse liver yielded over 2000 potential off-target cleavage sites. Targeted amplicon of a subset of 45 of these loci from the livers of mice treated with the gP/SpCas9 adenoviral vector showed that 19 of these sites showed significant levels of indel mutations (relative to negative control mice). Taken together, our studies show that SpCas9, when paired with a promiscuous gRNA, can efficiently generate off-target mutations *in vivo* (in some cases with very high frequencies). To our knowledge, this is the first demonstration that SpCas9 can induce substantial off-target mutations *in vivo*. More importantly, our validation of the VIVO approach shows that it has high sensitivity for identifying off-target mutations (with frequencies as low as 0.13%) *in vivo*. Thus, VIVO provides a highly effective strategy for evaluating the *in vivo* genome-wide specificities of CRISPR/Cas9 nucleases.

905. Real-Time MR Imaging and Pharmacokinetic Analysis of AAV Vector Delivery into the Cerebrospinal Fluid of Non-Human Primates

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Background: Therapies based on adeno-associated virus (AAV) technology have demonstrated significant potential to correct central nervous system (CNS) pathologies. Although parenchymal delivery has been by far the favored route, especially for AAV2, the potential advantages of intrathecal infusion of AAV have been recognized in particular for pediatric patients, too young to undergo AAV delivery using skull-mounted neuronavigation devices. In a number of studies in non-human primates, bolus injections of AAV vectors have yielded impressive but variable transduction of spinal cord, cortex and cerebellum. However, little attempt to optimize dosing parameters has been performed with any AAV serotype. **Objective:** The goal of this study was to understand the fluid dynamics of the AAV infusion in the cerebrospinal fluid (CSF) space, and therefore the distribution patterns of different delivery routes into CSF were analyzed by magnetic resonance imaging (MRI). In addition, levels of circulating AAV9 were analyzed to determine the clearance rate of injected vector over time. **Methods:** Thirteen Rhesus and Cynomolgus macaques were infused with the mixture of AAV9-human acid sphingomyelinase-HA (2.2×10^{13} vg/mL) and 1 mM gadolinium-based contrast agent into the CSF via 3 routes of delivery under MRI monitoring. Animals were randomly assigned to bolus cisterna magna infusion (also known as cerebellomedullary infusion or cisternal puncture) group (n=3, 1 mL/min), continuous cisterna magna infusion group (n=3, 1 mL/h), intrathecal lumbar infusion group (n=3, 1 mL/min), cisternal and lumbar combined infusion group (n=2, 1 mL/min), or bilateral lateral ventricle infusion group (n=2, 1 mL/min). Fluid dynamics of the vector in the CSF was analyzed by sequential acquisition of the gadolinium signal in a MRI scanner along the infusion. CSF was collected from cisterna magna at different time points (15 min, 30 min, 1 h, 6 h, 12 h, 24 h and 28 d after the end of infusion), and vector titer in the CSF was analyzed by quantitative PCR. All animals were safely recovered from anesthesia and did not show any clinical signs until the necropsy. Animals were sacrificed 28 days after the infusion. **Results:** Fluid distribution analysis revealed variability in the pattern

of vector dissemination regardless of the route of delivery. Quantitative PCR analysis of AAV9 in the CSF established its half-life to be approximately 4 h. Vector was almost undetectable 24 h after the end of infusion regardless of the delivery paradigm. Bolus cisterna magna infusion revealed slower clearance rate of the vector in the CSF, while intracerebroventricular infusion showed the fastest clearance rate, most likely due to a dilution effect. Similar patterns were found in the other experimental groups. **Conclusion:** Our data on vector distribution and clearance from CSF allow us to optimize technique for accessing CSF space, optimization of parameters such as rate and duration of vector infusion and frequency of AAV administration to the brain and spinal cord for pediatric and adult indications.

906. Gene Editing Specificity Assessment for EDIT-101, an LCA10 Therapeutic Candidate

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Leber's Congenital Amaurosis Type 10 (LCA10) is an early-onset retinal degeneration disease caused by mutations in the CEP290 gene. EDIT-101 is a therapeutic candidate designed to treat LCA10 patients that carry the most prevalent causative CEP290 mutation, called IVS26. EDIT-101 is an AAV5 vector packaged with DNA encoding the *S. aureus* CAS9 (SaCas9) protein, along with two guide RNAs. When expressed in photoreceptor cells, the dual gene editing machinery removes the IVS26 mutation and restores expression of the full length CEP290 protein. We expect this to improve photoreceptor function and bring clinical benefit to LCA10 patient harboring the IVS26 mutation. **Specificity** is a significant aspect for any gene editing therapeutic, as changes to DNA are permanent at the cellular level. A number of factors contribute to the specificity of EDIT-101, including: restricted expression of SaCas9 by sub-retinal injection of EDIT101, selection of the AAV5 serotype that shows tropism for photoreceptors, and the use of a photoreceptor-specific GRK1 promoter. In both mice and non-human primates (NHPs), specific transduction of photoreceptor cells by EDIT-101 was demonstrated using in situ hybridization (ISH) to the minus strand of the genome vector. Expression of SaCas9 protein, was shown to be photoreceptor specific by immuno-histochemistry (IHC). The **DNA-editing specificity** for the two SaCas9 guide RNAs on the human genome has also been characterized. DNA-editing specificity was assessed in two distinct phases: Discovery and Verification. In the Discovery Phase, several orthogonal methods were used to identify candidate off-target sites. These included: *in silico* analysis, biochemical cutting with the Digenome assay, and cellular DNA editing with the GUIDE-Seq assay. Each method has pluses and minuses, which will be discussed, and each produces a set of candidate off-targets that were pooled and brought forward to the next phase. In the Verification Phase, we assessed EDIT-101 editing at the candidate off-target sites using targeted Next Generation sequencing (NGS) panels in therapeutically relevant human tissue and photoreceptor cells, human retinal explants derived from cadavers - as well as cell lines. The panels achieved a sensitivity of ~0.1% editing detection for ~80% of the off-target sites. Results from these analyses will be presented. Further, the specificity approach defined by these analyses may be broadly applied to gene editing therapeutics.

907. Tracking AAV Gene Expression Non-Invasively in Small and Large Animals Using the NIS Reporter Gene: an Accelerated Approach to Gene Therapy Development

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Despite recent advances in gene therapy, significant challenges remain for directing therapeutic payloads to target tissues with high specificity *in vivo*. Viral vectors engineered for tissue-specific tropism and/or transgene expression are highly desirable to minimize off-target infection. However, the development of such "designer" vectors is hampered by conventional methods for assessing biodistribution (i.e. qPCR and IHC), which are extremely labor-intensive, time-consuming, and prone to sampling error. Additionally, these invasive techniques require large numbers of research animals, raising ethical concerns and increasing overall costs, particularly in large animal studies. Given these limitations of traditional biodistribution assays, we sought to develop a non-invasive and translational alternative for tracking gene therapies in living animals. The sodium iodide symporter (NIS) is a self-protein that concentrates iodide within cells of the mammalian thyroid and stomach. Beyond this physiological role, NIS can function as a reporter gene for non-invasive, quantitative, and tomographic SPECT or PET imaging via its ability to drive uptake of radiotracers. Unlike luciferase, NIS reporter gene imaging is high-resolution and translatable from small animal models to humans. To evaluate the utility of NIS for tracking gene therapies *in vivo*, we delivered an AAV9 vector expressing NIS (AAV9-NIS) intravenously into nude mice and monitored NIS transgene expression longitudinally within the same animals (Fig. 1). Using non-invasive PET imaging with F18-TFB, we detected NIS reporter gene expression with high resolution in the heart, liver, brown fat, and skeletal muscle beginning on day 7 post-administration. NIS expression peaked by day 14 and remained steady throughout the course of the study. Endpoint qPCR and IHC assays confirmed the sites of AAV9 transduction and correlated closely with NIS reporter gene imaging. In addition to these small animal studies, we and others have recently demonstrated the utility of NIS for tracking gene therapies in large animal models, including dogs and pigs (Moulay et al. 2015. *Mol Ther*; Hickey et al. 2016. *Sci Transl Med*). Combined, these data establish NIS reporter gene imaging as a non-invasive, translational alternative to traditional assays with the potential to expedite biodistribution studies of engineered viral vectors and, thereby, accelerate gene therapy development.

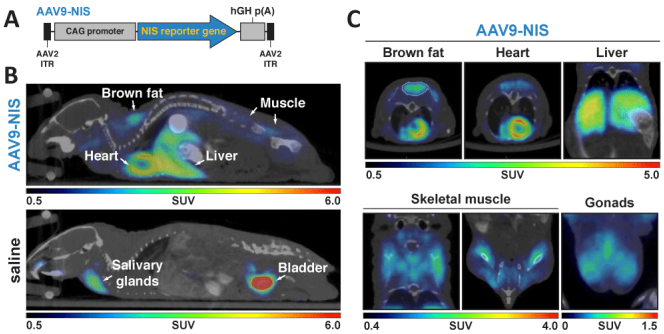

Figure 1. Non-invasive imaging of AAV9 transgene expression using NIS. **(A)** Genome organization of AAV9-NIS. **(B)** High-resolution PET imaging of NIS reporter gene expression in nude mice on day 23 post-administration of AAV9-NIS or saline. **(C)** NIS reporter gene expression at major sites of AAV9 transduction.

908. fMRI Derived Visual Field Measures Correlate Highly with Clinical Perimetry Assessments in Choroideremia Patients

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Introduction: Choroideremia (CHM) is an X-linked recessive form of hereditary retinal degeneration, which at advanced stages leaves only central islands of preserved retinal tissue. With excellent central vision, CHM patients have superior ability to maintain stable fixation, which makes them an ideal candidate for exploring the relationship between preserved residual vision and the retinotopic organization of visual cortex using Population Receptive Field (pRF) modeling of functional magnetic resonance imaging (fMRI) data. pRF modeling is a retinotopic mapping technique that uses a computational model of visual responses to provide an objective and efficient estimate of the subject's effective visual field. In this study, we apply pRF modeling to data from a group of patients with CHM and compare the results with their clinical static automatic perimetry (SAP) assessments. **Method:** Four molecularly confirmed CHM patients in early, mid and late disease stages and four matched controls completed comprehensive clinical evaluations including a standard SAP using a conventional 10-2 testing protocol and horizontal sensitivity profile that extended to 10° from central fixation in light and dark-adapted conditions. All participants also underwent monocular fMRI/pRF scans using a 3.0T research MRI system equipped with a 12-channel head coil and MRI compatible video goggles. Participants were asked to fixate on a central target while a bar aperture containing 100% contrast checkerboards systematically traversed their visual field. The pRF model outputs estimated diameter and center location (x, y) of each voxel's pRF. Initially, visual field coverage maps were computed using all significant voxels. These coverage maps demonstrate clearly the diminished peripheral representations in CHM participants. Next, the relationships between patient's residual vision and estimates of pRF were established by spatially resampling the visual field coverage to match the SAP 10x10 matrix and populating each cell with its mean pRF value. Additionally, pRF derived horizontal eccentricity for each patient and eye were computed by calculating the mean pRF value in linearly spaced column vectors that spanned the full width in X-direction and first four degrees along the Y-axis visual field, with matching cells containing the clinically measured horizontal eccentricity. **Results.** The spatial distribution and strength of pRF estimates correlated positively and significantly with clinically measured SAP in the majority of CHM participants. Importantly, the positive relationship between clinical and pRF measured visual fields increased with increasing disease progression. The pRF estimates of the extent of the visual field (eccentricity) also highly correlated with clinically measured extent of the fields by SAP. **Conclusions.** Clinical evaluations of visual function in participants with CHM are well characterized by the spatial distribution and strength of pRF estimates using a single fMRI experiment. These preliminary results show that pRF driven visual field and eccentricity measures have the potential to be considered as an efficient and objective measure to complement current ophthalmic evaluations. Furthermore, this short 3-minute pRF/fMRI acquisition could offer an additional metric to assess the efficacy of future retinal interventions to restore vision.

909. ViralSeq-Toolkit: A Computational Toolkit for Characterization and Analysis of Viral Vectors

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Recent advancements in gene and immune therapies and approval of products for retinal dystrophy, lipoprotein lipase deficiency and cancer, reinforce the promises of these therapies to treat challenging disorders. Viral vectors have emerged as a potential tool for efficient and stable genetic modification of cellular genome and their progeny. In-depth evaluation of efficacy and safety of viral vectors is crucial to ensure optimum therapeutic benefits and elucidate possible harmful effects e.g., immunogenicity and genotoxicity. We present here ViralSeq-Toolkit for comprehensive characterization and analysis of viral vectors. Our toolkit is comprised of specific analysis modules: starting from the characterization of purity of viral vector preparations to post-administration efficacy and safety evaluation in pre-clinical and clinical settings. The first module of ViralSeq-Toolkit allows identification of potential contaminants in vector preparations, distribution of each contaminant and fragments size estimation. The second module performs analysis of vector integration sites within the host genome to characterize population of transduced cells and clonal contribution. The obtained set of genomic integration sites is annotated according to nearby gene features and potential oncogenic relevance. An additional mode allows the analysis of internal breakpoints distribution of viral vectors. Each module of toolkit is designed in a way to handle analysis of multiple vectors in a concurrent manner. This user-friendly toolkit is implemented in Linux and Perl. ViralSeq-Toolkit combines the functionalities to cover a range of gene therapy data analysis

requirements and can be efficiently employed on DNA and RNA whole genome and targeted sequencing high throughput data. It additionally has broader implications in analyzing next-generation sequencing data for presence of viral and non-viral contaminants, analysis of data from insertional mutagenesis screens and investigation of viral cancers. In future, it will be extended further for immune repertoire analysis (T and B cell receptors) to provide a platform for integrative analysis of viral vector insertion and distribution patterns with immune status.

910. Quantitative Relationship between Vector Copy Number and Transduction Efficiency Using a Modified Poisson Equation Reveals Factors Controlling Transduction Uniformity

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Assessment of lentiviral vector (LVV) transduction efficiency typically relies on measuring both a composite vector copy number (VCN) across a population of cells (expressed as VCN per diploid genome), and the percentage of transduced, or LVV-positive (%LVV+) cells. The relationship between VCN and %LVV+ cells is not well understood except that the two readouts are positively correlated. Plotting %LVV+ cells across a range of VCN values produces an asymptotic curve that can be fitted with a modified Poisson distribution. The fitted equation quantifies both the fraction of cells that do not transduce with LVV, forcing the asymptote below 100% LVV+ cells, and transduction uniformity: the skewing away from the expected normal Poisson distribution of LVV integrations among cells. Transduction uniformity was found to be high in cell lines, but interestingly, it was still not completely uniform, and skewed away from a Poisson distribution. Cell line transduction uniformity was not impacted by multiplicity of infection (MOI), cell density, confinement of LVV to a retronectin-coated surface, or addition of valproic acid to loosen chromatin. Transduction uniformity was lower in primary human CD34+ cells and T-cells, and the lower transduction uniformity in primary cells was mimicked by transducing a mixture of cell lines with different permissibility to transduction, indicating that transduction non-uniformity is due to varying permissibility among subsets of cells. A refined manufacturing process significantly reduced the donor-to-donor variability in transduction uniformity of CD34+ cells. As with cell lines, the MOI was found to determine the placement of data points on the VCN vs %LVV+ curve, but did not affect the relationship between VCN and %LVV+ cells. Altogether, this study demonstrates that both in cell lines and in primary cells, the fraction of non-transducible cells is small, and the relationship between VCN and %LVV+ cells deviates from the Poisson distribution mostly due to quantifiable transduction non-uniformity, where LVV copies are disproportionately sequestered by a subset of cells. Once the relationship between VCN and %LVV+ cells is established, the two measurements are redundant, and the MOI needed to reach a specific point on the VCN vs %LVV+ cell curve depends on cell-intrinsic factors that are donor-dependent for primary CD34+ cells.

911. Sensitive QPCR Approach to Monitor CAR T-Cell Persistence and Expansion in Patient's Samples

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Expansion of CAR T cells is essential to confirm tumor encounter and proliferation upon recognition of targeted -tumor cells in patients treated with CAR T cells. The amplitude of the expansion in various body compartments is a tool to survey early responses and CAR T cell-induced toxicities. Conventional assays used by investigators to evaluate expansion encompass flow cytometry to detect transduced T cells and qPCR to measure the number of vector copies (VCN) per ug of genomic DNA or per white blood cell (WBC) count. In our facility, a dual target amplification of vector and albumin gene assay is used to evaluate transduction rate in CAR T cell products and CAR T cell persistence in patient's samples with some time points tested by flow cytometry as well. Upon retrospective analysis of flow cytometry and qPCR paired data sets, we uncovered poor correlation between these methods when less than 10% of the cells are positive by flow cytometry. Upon further analysis, the dual target qPCR assay displays a restricted range of positive results due to a competition between vector and albumin sequence detection. Our objective was to evaluate the performance and the feasibility of a modified assay standardized on sample volume, requiring neither quantification of extracted DNA neither determination of WBC by mean of cell counting or albumin gene amplification. Pre- and post-infusion samples were tested with current dual target assay standardized on 200ng DNA input from WBC extract and with modified single (vector sequence) target assay standardized on 25% of the extracted volume from 200uL of whole blood. Blood samples (n=163) were tested returning positive results for 90 (55%) with the modified assay and 18 (11%) for the dual target assay. The lower limits of detection and quantification were 50 and 100 copies per mL of blood and 2 and 5 copies per 200ng of DNA, respectively. Both returned 9% of positive in the undetermined range. The increased sensitivity of the new assay restored 100% agreement (n=14) with positive flow cytometry results. Overall the modified assay showed high performance and sensitivity increased five times with a simplified process allowing vector quantification from 200uL of blood. An internal quality control will be further included to validate blood samples returning a negative result.

912. Acquisition of Genome-Edited iPSC Clones with Low Off-Target Editing by Efficient Screening System Utilizing NGS Analysis

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The target specific genome editing in induced pluripotent stem cells (iPSCs) holds promise in wide variety of fields. Single base substitution using single-stranded oligo DNA nucleotides (ssODNs) can be used for

introducing and/or correcting mutations to generate human disease model for drug discovery and elucidation of diseases. Precise mutations can be achieved by homology-directed repair, but the efficiency is usually very low. In addition to the improvement of genome-editing efficiencies, the optimal culture system for single cell cloning to promote expansion of the clones and maintain the pluripotency throughout the whole process is crucial in iPSC genome-editing. Previously, we have established a highly efficient system to obtain genome-edited cells with single-base substitutions using Cas9 RNPs delivered with optimized ssODNs, without using additional components that might cause unwanted effects on the cells. Furthermore, off-target editing such as undesired insertions and deletions (indels) at the target site or other sites with high sequence homology, is still a major issue. It is essential to perform efficient screening of the correctly edited clones without any undesired editing. In this study, to select the optimal culture system for genome editing, we have compared the several culture systems for iPSCs in single cell cloning step, and found the Cellartis® DEF-CS™ culture system was superior in expansion of the clones. Moreover, all the expanded clones maintained their pluripotency, while many of the expanded clones lost the expression of the pluripotency markers in other culture systems. We also have developed an efficient screening system for precisely edited clones using the next generation sequencer (NGS). The genome-edited cell populations were seeded and cultured at the density of 5-20 cells/well, and the amplicon sequence analysis was performed in each well using Illumina's MiSeq® system. Using this screening system, we could reliably detect the wells containing the amplicons that possess only the desired mutation, and could also analyze the amplicons from other targets sites. Simultaneous analysis of both on- and off-target sites enabled screening of the cell population having a low off-target risk. Cytochromes P450 (CYP) play a major role in drug metabolisms, and certain single nucleotide polymorphisms (SNPs) have a large effect on CYP activity related to the efficacy and adverse effects of drugs. Nowadays, the iPSC-derived hepatocytes are expected to be an alternative to primary hepatocytes in drug toxicity test. The genome edited iPSCs with desired SNPs at CYPs could be the useful sources in drug screening, then we have tried to establish the genome-edited iPSC clones with the specific SNP at CYP2C9*3. However, having the high sequence homology to other CYP genes, obtaining the clones having only desired SNP without any off target editing will be almost impossible. Using the established screening system by the simultaneous NGS analysis of amplicons from multiple sites, we could obtained the clone having only the intended SNP in CYP2C9*3. During the whole process of genome editing and screening, the iPSCs were cultured in Cellartis® DEF-CS™ culture system, and the obtained clone conserved its pluripotency, and could differentiated into hepatocytes using Cellartis® iPS Cell to Hepatocyte Differentiation System.

913. qPCR Method Development and Validation for Analysis of Supercoiled dMAb Plasmid Biodistribution in Monkeys

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Advances made in the delivery and construction of non-viral DNA vectors has led to an increase in the use of plasmid DNA for the in vivo production of antigens and proteins. The DNA vector in this study produces a monoclonal antibody (dMAb) of the Ebola virus. Like other compounds introduced into the body, these supercoiled plasmids still carry potential safety concerns to human health after administration. Assessment of risk begins with the analysis of physiological biodistribution and persistence within the system utilizing quantitative polymerase chain reaction (qPCR) assays. One of the more prominent challenges of qPCR is the ability to robustly and accurately quantify supercoiled plasmid DNA. Increased tension within these circular DNA can reduce primer binding efficiency thereby increasing assay variation and decreasing sensitivity. These effects can hinder the ability to validate the qPCR assays necessary for evaluating the biodistribution of the plasmid vectors in dosed animals. In this study, we developed and validated a qPCR assay to effectively quantify supercoiled plasmid DNA in the presence of 1µg of Rhesus monkey genomic DNA. We tested several qPCR additives such as dimethylsulfoxide (DMSO), BSA, and pluronic F-68 in various concentrations and combinations to determine their effects on specificity, sensitivity, and variation of the assay. Here we show that addition of both non-ionic detergent, pluronic F-68, and DMSO improve amplification efficiency and increase specificity. Inter-assay precision and reproducibility were evaluated over six separate qPCR runs. Over those six runs, we obtained both and intra- and inter-assay Ct %CV of 2.0% or less at each standard level to 50 copies. In addition to those variables, twenty quality control samples were prepared with each run to analyze accuracy of the assay. Out of 120 quality control samples, 92 percent met our recovery accuracy criteria within ±25% of the relative error.

914. Effects of Lentiviral Vector Administration on a DEN/CCl4-Induced Liver Injury Mouse Model

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We have generated a lentiviral vector (LV) expressing fumarylacetoacetate hydrolase (LV-FAH) intended for *in-vivo* gene therapy for hereditary tyrosinemia type I (HT1). To develop this vector for consideration for potential human clinical use we have evaluated general- and specific hepato-toxicity of a single intravenous dose in wild-type mice and mice subsequently exposed to *N*nitrosodiethylamine (DEN) and carbon tetrachloride (CCl_4) to induce liver injury. Briefly, groups of

20 male mice were dosed with vehicle, DEN/CCl4, LV-FAH (10^9 TU/mouse), or the combination of LV-FAH and subsequent DEN/CCl4 over a 109-day study period. All groups were evaluated for general toxicology endpoints, including body weights, cage side observations, serum chemistry, hematology, and gross pathology. Hepatotoxicity was specifically interrogated with routine histopathology and expression of alpha fetoprotein, a biomarker associated hepatocellular carcinoma. Although there were no changes in observational data associated with any treatment group, both the DEN/CCl4 induction and LVV-FAH caused slight, non-additive, statistically significant decreases in body weight gain over the course of the study (4.2x gain for controls compared to 3.2-3.7x for treated groups). DEN/CCl4 resulted in expected liver toxicity and, alone or in the presence of LV-FAH, was associated with heart weight decreases (0.85-0.81x control, respectively) and spleen weight increases (1.06-1.12x, respectively). LV-FAH alone or with DEN/CCl4 was associated with reduced kidney weights (0.93-0.91x, respectively) with some animals exhibiting mild increases in BUN and/or creatinine. In the liver, exposure to DEN/CCl_4 alone or with LV-FAH was associated with elevations in AST (2.3-2.7x, respectively) and ALT (3.2-4.2x, respectively), but only the combination group demonstrated increases in liver to body weight ratio (4.5x compared to 4.1x). Histologically, DEN/CCl_4 showed diffuse hepatocellular hypertrophy and/or hyperplasia, karyocytomegaly, biliary stasis, and bridging fibrosis/fibroplasia. However, the combination of DEN/CCl_4 and LVFAH was associated with an increased severity of these findings, with higher incidence of bridging fibrosis and advancing to hepatocyte degeneration and necrosis. There were no hepatocellular tumors observed in any group tested; however, DEN/CCL4 caused increases in alpha fetoprotein levels that were exacerbated in the context of pretreatment with LV-FAH. These results indicate that systemic LV-FAH administration was unequivocally safe, with no adverse effects at this therapeutic dose in any parameters tested. However, in the context of a chemically injured liver model, pretreatment with LV-FAH was associated with increased frequency and/or severity of toxicity. These results support therapeutic development of *in vivo* lentivirus administration, and further, clearly show that both healthy and disease models must be evaluated.

915. Establishment of a Non-Invasive and Efficient *In Vitro* Assay System for Exon Skipping Therapy

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<Background> Duchenne muscular dystrophy (DMD) is a severe muscle disorder characterised by mutations in the *DMD* gene. Recently, we have completed a phase I study in Japan with systemic administration of the Morpholino antisense NS-065/NCNP-01 for exon-53 skipping in DMD aimed to achieve a highly favourable safety profile, promising pharmacokinetics and efficacy. *In vitro* assays using DMD patient-derived cells are indispensable before the clinical trial, and we have established an *in vitro* assay existing system based on the fluorescence-activated cell sorting (FACS)-aided *MyoD*-transduced fibroblasts. <Purpose> To newly establish a non-invasive *in vitro* assay capable of efficient evaluation of exon-skipping on patients' urine-derived cells (UDCs). <Methods and Results> We developed a retroviral doxycycline-regulated inducible *MyoD* expression system, which enables puromycin selection cell sorting instead of FACS and regulation of cell proliferation/differentiation after *MyoD* transduction. By using the new system, we converted UDCs to myogenic cells. Following Morpholino transfection into human UDCs, we observed highly efficient exon skipping and dystrophin protein recovery. We also found that UDCs express CD13 and CD90, but not CD105, a mesenchymal stem cell marker, suggesting that UDCs are tubular proximal epithelial cells with low stemness. Furthermore, histone methyltransferase inhibitor significantly promotes the direct-reprogramming of UDCs into myogenic cells, leading to a shortening of the cell incubation period of the assay. <Conclusions> We successfully established a non-invasive *in vitro* assay system for exon skipping using *MyoD*-transduced UDCs.

916. Evaluation of a Biological Potency Assay for an AAV2.AADC Vector Used in the Treatment of Parkinson's Disease

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VY-AADC (AAV2.AADC) is a gene therapy product that is delivered to the brain and is currently in clinical trials for the treatment of Parkinson's Disease. The vector shows promise in increasing AADC levels in the target brain regions and improving the clinical response to levodopa in Parkinson's Disease patients. In order to better characterize the product and assess lot-to-lot consistency, a potency assay was developed to determine the effectiveness of AAV2.AADC vectors relative to a vector reference standard. The method was tested for linearity/range, precision, repeatability, and specificity. Evaluation of the data demonstrates that this method is suitable for assessing the relative potency of AAV2.AADC gene therapy vector products.

917. Use of Novel Transgenic NIS Reporter Rats for Longitudinal Tracking of Fibrogenesis by High-Resolution Imaging

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Introduction: Type 1 collagen is major component of fibrotic scar tissue formed during wound repair in a process known as fibrogenesis. Inadequate fibrogenesis leads to poor healing and delayed tissue regeneration. However, excessive fibrogenesis causes organ fibrosis, which is a common cause of liver, lung, heart or kidney failure. Thus,

fibrogenesis is a target for the development of new drugs that seek to maintain a state of regeneration homeostasis that is not detrimental to the human body. To date, connective tissue synthesis during both normal and pathological conditions and its modulation by drug therapy have been difficult to study in living animals. Collagen synthesis following injury has been classically studied by biochemical and immunohistochemical analysis of tissues postmortem. However, this method does not allow for longitudinal analysis of a single animal and has highlighted the variability of wound healing/fibrogenesis kinetics between animals. Previous studies have created transgenic animals expressing various collagen promoter-driven reporter genes (CAT, β-gal, GFP, and luciferase) but these models cannot be used for live monitoring of fibrogenesis or resulted in poor spatial resolution imaging. Therefore, creation transgenic animals that utilizes collagen promoter-driven sodium iodide symporter (NIS) reporter gene in conjunction with PET or SPECT nuclear imaging is an attractive alternative technology for development of a novel fibrogenesis model that can be tracked non-invasively overtime. **Methods:** To non-invasively monitor collagen synthesis, an indicator of fibrogenesis, after tissue injury we generated transgenic reporter rats expressing the human sodium iodide symporter (NIS) driven by the rat collagen type-1 alpha-1 (Col1α1) promoter. NIS radiotracer uptake was monitored longitudinally using high resolution SPECT/CT or PET/CT following unilateral rotator cuff (RC) injury. **Results:** Radiotracer uptake was first detected in and around the injury site day 3 following surgery, increasing through day 7-14, and declining by day 21, revealing for the first time, the kinetics of Col1α1 promoter activity in situ. Differences in the intensity and duration of NIS expression/collagen synthesis between individual RC injured Col1α1-hNIS rats were evident. Additionally, we show that daily subcutaneous dexamethasone treatment of our Col1α1-NIS transgenic rats with RC injury is able to delay the kinetics of the Col1α1 promoter activity. **Conclusions:** For the first time, we demonstrate longitudinal imaging of Col1α1 promoter activity with exquisite resolution as a model of inflammation and fibrogenesis that can be modulated by a known inhibitor of inflammation and collagen synthesis. This approach of in vivo monitoring of fibrogenesis using living NIS reporter transgenic rodents will facilitate research and drug development in many applications.

918. Safety and Bio-Distribution of Freshly Thawed Human Mesenchymal Stem Cells in Healthy Nude Rats

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Introduction: Mesenchymal stem cells (MSCs) are multipotent cells that have been shown to exert important immunomodulatory effects in both acute and chronic inflammatory diseases. However, in a severe acute inflammatory condition like septic shock, immunomodulatory cell therapy may be most efficacious when administered early within the first several hours when inflammation is most severe. Therefore, an "off-the-shelf" cryopreserved, allogeneic cell product that can be thawed prior to infusion to the patient is best suited for this purpose. In this study we evaluated the safety and bio-distribution profile of the freshly thawed human MSCs in healthy nude rats. **Materials & Results:** Cryopreserved bone marrow derived human MSCs were thawed and diluted prior to infusion to healthy nude rats (Charles River). The cells were prepared to a target concentration of 2.8 million cells/mL with a target dose of 1.2 million cells/animal (approximately equivalent to 3 million cells/kg). A single dose of freshly thawed MSCs or vehicle control was injected into healthy nude rats followed by a 28 day observation period prior to blood and tissue collection at the end of study. During this study, wellness assessments were performed, including measurement of all deaths that occurred by the end of the observation period, and body weight measurements taken pre-treatment and weekly post-treatment until the end of the observation period. Samples collected at the end of the study were used to measure clinical chemistry parameters (AST, ALT, GGT, LDH, creatinine, bilirubin, urea), perform histopathology examination (brain, heart, right lung, whole liver, spleen, both kidneys, and both gonads [testes or ovaries]), measure organ weight and conduct bio-distribution analysis. There were no MSC-related adverse effects noted on mortality or body weight. MSC infusion was not associated with adverse clinical chemistry changes, change in organ weight nor histopathology from any of the examined organ tissues. Bio-distribution analysis (via quantitative PCR) also did not detect a signal of human MSCs from organ tissues collected at 28 days post-infusion. **Conclusion:** Our results suggest that a single dose of freshly thawed human MSCs in healthy nude rats was well tolerated with no changes in body weight, selected clinical chemistry parameters, or histopathology of examined tissues as compared to the vehicle control. The bio-distribution showed no detectable signal of human MSC at 28 days post infusion, suggesting lack of engraftment of the injected cells.

Synthetic/Molecular Conjugates and Physical Methods for Delivery of Gene Therapeutics II

919. Non-Viral Gene Therapy for Brain Tumors via Convection Enhanced Delivery (CED)

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Although gene therapy has shown a powerful strategy for Glioblastoma (GBM) treatment in preclinical studies, its effectiveness has yet to be achieved in humans due in large part to an inability to achieve widespread distribution in the brain. We recently demonstrated that sub-120 nm nanoparticles can rapidly diffuse in the brain extracellular matrix (ECM) if they are well-coated with hydrophilic and neutrally charged polyethylene glycol (PEG), providing improved distribution of therapeutics. Convection enhanced delivery (CED) can further enhance the volume of distribution in the brain by providing a pressure gradient during intracranial administration. However, CED is unlikely to provide a significant benefit if particles

are trapped in the brain parenchyma due to adhesive interactions and/or steric obstruction. Here we developed a DNA-Brain Penetrating Nanoparticle (**BPN**) system (<100 nm) with a neutral surface charge and long shelf-life. In addition, we established a 3D-tumor spheroid model that shows direct correlation with the particles performance *in vivo*, which could be beneficial for screening different types of gene vectors, including PEGylated ones, at a smaller scale providing faster results. After evaluating the DNA-BPN *in vitro*, *ex vivo*, and *in vivo*, it was proved that the particles ability to transfect and penetrate the brain is dependent on the surface's PEG density. When administered by CED into the rat striatum, highly PEGylated BPN, named **BPN^{H}**, distribute throughout and provide broad transgene expression in healthy (Figure 1) and F98 GBM tumor brains (Figure 2), without vector-induced toxicity. The use of these BPN^{H}, in conjunction with CED, offers an avenue to improve gene therapy for GBM. Figure 1. Transgene expression of ZS-Green following CED administration of DNA NP in rodent striatum.

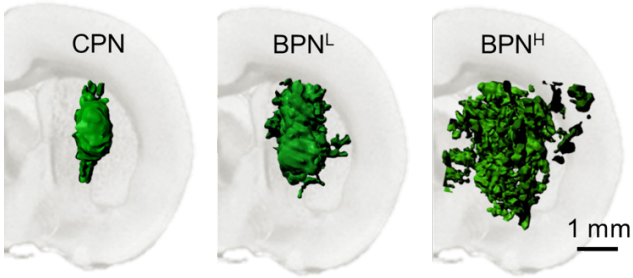
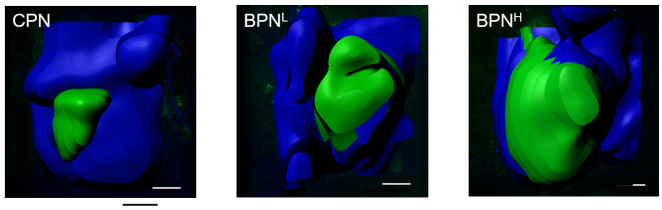


Figure 2. Transgene expression of ZS-Green in F98 GBM tumor-brain (DAPI).



920. Treatment of Hemophilia a Using Factor VIII Messenger RNA Lipid Nanoparticle

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Deficiency of Coagulation Factor VIII (FVIII), a glycoprotein that participates in the intrinsic pathway of the coagulation cascade, or its function leads to a serious bleeding disorder, hemophilia A. The current treatment for hemophilia A patients is replacement therapy of FVIII protein, which is costly and inconvenient. Development of a nonviral gene therapy such as delivery of mRNA using lipid nanoparticles (LNPs) can dramatically change the paradigm. mRNAs can be therapeutic agents as they can be delivered, translated, and processed accurately post-translation in cytoplasm to avoid risk of gene mutagenesis and to produce biologically, fully functional factors. In addition, advancement of LNP technology has offered a biocompatible, scalable, and less immunogenic delivery system. Here, we explored the therapeutic effects of FVIII mRNA via LNP delivery in hemophilia A murine models. We first investigated the delivery efficiency of our LNPs by using a luciferase reporter mRNA (Luc mRNA) and infused the Luc mRNA LNPs intravenously into wild type C57BL/6 mice. Transgene expression was examined using a bioluminescent *in vivo* imaging system 7 hours after injection. Biodistribution of luminescence showed luciferase expression occurred mainly in the liver, indicating tissue specificity. To assess biocompatibility of the LNP composition, LNP/Luc mRNA was injected daily for 5 consecutive days into C57BL/6 mice. Livers were harvested staining for CD4 and CD8 expression showed minor presence of lymphocyte infiltrate. We then examined the therapeutic potential of FVIII mRNA LNP for treatment of hemophilia A. Three groups of hemophilia A mice were injected with LNPs carrying three different variants of human FVIII mRNA. Hemophilia A mice injected with FVIII variants showed FVIII activity 12 hours post-injection, as a measure of clotting activity. Single dose and dose response studies in mice showed efficacy at 24hrs that was maintained up to 72hrs post injections, suggesting a potential therapeutic regimen. In conclusion, our data demonstrated specific LNPs can efficiently deliver mRNAs into the liver to achieve high level gene expression without inducing liver damage or immune responses. Furthermore, hemophilia A mice treated by FVIII LNPs produced significantly increased clotting activity more than 3 days after single administration. Our study established a safe and effective platform of new mRNA therapies for hemophilia A.

921. Precise Enzymatic Cleavage Sites for Improved Bioactivity of siRNA Lipopolyplexes

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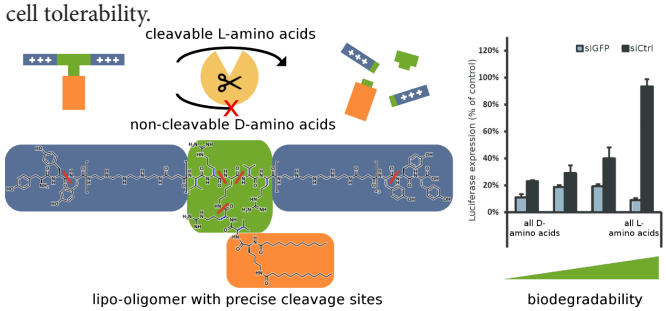
Sequence-defined cationic lipo-oligomers are potent siRNA carriers based on stable electrostatic and hydrophobic polyplex formation and endosomal membrane destabilization. Such nucleic acid carriers should be readily biodegradable to ensure high systemic tolerability and low accumulation of potentially harmful amphiphilic oligomers *in vivo*. After endocytosis, intracellular triggers like changes in pH and redox potential or occurrence of enzymes can, on the one hand, facilitate the release of the encapsulated cargo and on the other hand enable a rapid excretion of cleavage products with reduced cytotoxicity. A library of sequence-defined lipo-oligomers containing natural and artificial amino acids with precisely introduced cleavage sites was synthesized by solid-phase supported synthesis. The degradability of the structures was tailored by introducing either short cleavable L-amino acid sequences, non-cleavable D-amino acid linkers or varieties of both. The lipo-oligomers were incubated with the protease cathepsin B to simulate lysosomal degradation. Cleavage products were identified by MALDI-TOF mass spectrometry. The effect of improved intracellular cleavability on cell tolerability was studied *in vitro* by transfecting Huh7- and DU145-eGFPLuc cells. Precise positioning of enzymatic cleavage sites between a lipophilic diacyl domain and an ionizable oligocationic siRNA binding unit strongly enhanced the degradation of the carrier. The cell tolerability was significantly improved for highly degradable structures, as no reduction of luciferase expression by siCtrl control lipoplexes was observed compared to less or non-

degradable carriers. Therefore, incubation of synthetic nucleic acid carriers with lysosomal enzymes like cathepsin B can be a meaningful assay to evaluate their degradability as an important characteristic for cell tolerability.



Abstract Figure: *Precise integration of enzymatic cleavage sites into lipo-oligomers improves cell tolerability after siRNA transfection.*

922. Hur Targeted Therapy Induces the Immune-Suppressor PD-L1 (B7-H1): An Escape Mechanism in Lung Cancer

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HuR is an mRNA-binding protein that post-transcriptionally regulates the expression and stability of several oncoproteins. We recently demonstrated tumor-targeted HuR siRNA nanoparticle (NP) delivery significantly suppressed lung tumor growth both *in vitro* and *in vivo*. While our study results were exciting, we serendipitously identified siHuR-NP therapy modulates PD-L1 expression in lung cancer cells. Expression of Programmed Death Ligand 1 (PD-L1; B7-H1, CD274) was detected in human lung cancer cell lines. siRNA-NP treatment increased PD-L1 expression in H1299, A549, HCC827, and H1975 cell lines. Increase in PD-L1 was confirmed by western blotting, and flow-cytometry. Cell fractionation studies showed PD-L1 expression increased on cell membrane. Molecular studies showed HuR transcriptionally regulated PD-L1. Overexpression of HuR reduced endogenous PD-L1 expression in A549 and HCC827 cell lines indicating an inverse correlation between HuR and PD-L1. Immunohistochemical staining of human lung tissue microarray (TMA) for both HuR and PD-L1 showed a strong inverse correlation and concurred with the *in vitro* cell line study results. To understand the functional importance of PD-L1 upregulation by HuR-NP treatment, we co-cultured activated T-(Jurkat) cells with tumor cells and measured PD-L1 expression on tumor cells. Co-culturing of tumor cells with Jurkat cells resulted in increased PD-L1 expression on tumor cell surface while increased apoptosis in T-cells. Interestingly, co-culturing of HuR-NP-treated tumor cells with T-cells resulted in reduction in PD-L1 expression in tumor cells and increase in T-cell survival. Analysis for interleukin (IL)-2 showed a marked increase in the supernatant collected from HuR-NP-treated tumor cells suggesting that the increase in T-cell survival observed in co-culture studies is likely supported by the IL-2. Our data shows HuR-NP-mediated upregulation of PD-L1 could attenuate the efficacy of HuR-based therapy. In conclusion, a better understanding of the HuR/PD-L1 interaction will provide a rationale for combining HuR-NP with PD-L1 inhibitors for improving therapeutic outcomes.

923. Liver Expression of Metabolically Stabilized Double Stranded mRNA

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Utilization of mRNA for gene transfection offers advantages over DNA delivery because it circumvents the need to penetrate the nucleus for protein expression. In comparison to DNA, however, the half-life of translatable mRNA in a cell is substantially shorter. The present study prepared double stranded mRNA possessing different lengths of reverse strand and tested these for expression in vivo. The level and persistence of expression of Luciferase mRNA possessing a poly(A) tail ranging in size from thirty-two to over one hundred adenosine residues was examined using hydrodynamic dosing and monitoring of luciferase expression over time. Protection of mRNA with an 80-adenosine tail by hybridization with a complementary strand decreased the gene expression in vivo by ten-fold due to denaturation of the 3' and 5' UTR. Addition of non-coding nucleotides after the tail also decreased luciferase expression. However, hybridization of an antisense DNA oligonucleotide near the poly(A) tail allowed RNase H-mediated removal of the non-coding nucleotides and reactivation of the mRNA as demonstrated by gel electrophoresis and hydrodynamic dosing.

924. Harnessing exosome-Based Approaches for Development of Biomarkers and Novel Therapeutics in Spinal Muscular Atrophy

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Affecting 1 in 6,000 births, spinal muscular atrophy (SMA) is the most common cause of death in children due to a genetic disorder. SMA is caused by reduced levels of the survival of motor neuron (SMN) protein, resulting in loss of motor neurons and atrophy of muscle, as well as several other tissues and organs. While Spinraza, an antisense oligonucleotide, was approved for the treatment of SMA, approximately only 40% of children respond to treatment and there is currently a lack of an effective, low-cost biomarker to measure disease or treatment progress. Cells release a variety of protein and nucleic acid-loaded vesicles including exosomes. Serum-derived

exosomes can be an indicator for an assortment of disease states including various forms of cancer and neurodegenerative diseases. Furthermore, exosomes can deliver their contents from one cell to another, suggesting they may be a viable therapeutic delivery vehicle. Exosomes were therefore investigated as a potential biomarker and therapeutic for SMA. Exosomes were isolated from a cell culture and animal models of SMA, and human patient serum using Exoquick or differential centrifugation. Size and concentration of exosomes were determined using Zetaview nanoparticle tracking and electron microscopy. SMN protein was found within exosomes, and the levels of exosomal SMN protein was reflective of the disease state of the cell, animal or patient. Furthermore, all models of SMA, had higher levels of circulating exosomes within media or serum in comparison to their healthy controls, suggesting that SMN-deficient cells have a potential deficit in exocytosis or endocytosis machinery. We also show that the level of SMN protein contained in exosomes is dramatically elevated in cell lines engineered to overexpress the protein. SMN-loaded exosomes effectively delivered SMN protein to SMN-deficient cells in culture, in a dose and time dependent manner. In conclusion, SMN protein is released from cells in exosomes, and the levels of exosomal SMN protein and concentration of exosomes are dependent on the disease state of the cell from which they were derived. Importantly, SMN-loaded exosomes can deliver SMN protein to SMN-deficient cells, suggesting a novel approach to treat SMA.

925. Biocompatible Highly Condensed Plasmid DNA by Mono-Ion Complexes for In Vivo Diffusive Gene Delivery

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Introduction: In this study, we consider that the decrease in the transfection activity of polycations *in vivo*, as compared with that *in vitro*, results from their polyion complex (PIC) formation. Namely, owing to crosslinking between polycations and plasmid DNAs (pDNAs), the disadvantage of *in vivo* gene delivery mainly stems from the difficulty in controlling the properties of the resulting polyion complex at the nanoscale size. To avoid the crosslinking by polycations, we have establish the concept of the "mono-ion complex (MIC)" formation between pDNA and a mono-cationic biocompatible polymer [1-3]. Methods: To form the hydrogen bond with pDNA, omega-amide-pentylimidazolium end-modified PEG, that is, APe-Im-PEG, has been synthesized. For the confirmation of the MIC formation, agarose gel electrophoresis was performed, followed by the measurements of circular dichroism (CD) and melting temperature (Tm) of double-strand DNAs. The resulting MICs were injected into the skeletal muscle by intramuscular administration in mice, followed by reporter gene expression assay. Results and Discussion: Here, our original concept of the MIC between pDNA and a mono-cationic PEG has been stabilized by hydrogen bond formation. To form the hydrogen bond with pDNA, as shown in Figure 1, ω-amide-pentylimidazolium end-modified PEG, that is, APe-Im-PEG, has been synthesized. Agarose gel retardation assay and circular dichroism measurement have revealed that the MIC between pDNA and APe-Im-PEG has been stabilized by the hydrogen bond between pDNA and the ω-amide group, and that the stable MIC has surprisingly further migrated into gel, as compared with naked pDNA. The rise of melting temperature suggests that the specific hydrogen bond forms between an adenine-thymine base pair and the ω-amide group (Figure 1). As shown in Figure 2, the resulting pDNA MIC with APe-Im-PEG has enhanced the gene expression of enhanced green fluorescent protein (EGFP) in the broad area of skeletal muscle by intramuscular administration in mice, as compared with a naked pDNA or pDNA PIC from linear poly(ethyleneimine) (jet-PEI). These results suggest that the pDNA MIC is diffusive *in vivo* administration site, as compared with pDNA PIC. Our methodology for MIC stabilization by a ω-amide group is expected to offer superior suprarmolecular systems to those by ubiquitous PICs for *in vivo* diffusive gene delivery. Conclusion: The pDNA MIC with APe-Im-PEG has enhanced gene expression by intramuscular administration in mice, and is considered to be diffusive into in vivo administration site. References: [1] S. Asayama, A. Nohara, Y. Negishi, H. Kawakami, Biomacromolecules, 15, 997-1001 (2014). [2] S. Asayama, A. Nohara, Y. Negishi, H. Kawakami, Biomacromolecules, 16, 1226-1231 (2015). [3] S. Asayama, A. Nohara, H. Kawakami, Y. Negishi, WO 2014/148378 A1 (PCT/JP2014/56873). Acknowledgement: This work was partially supported by JSPS KAKENHI Grant Number 16H03183.

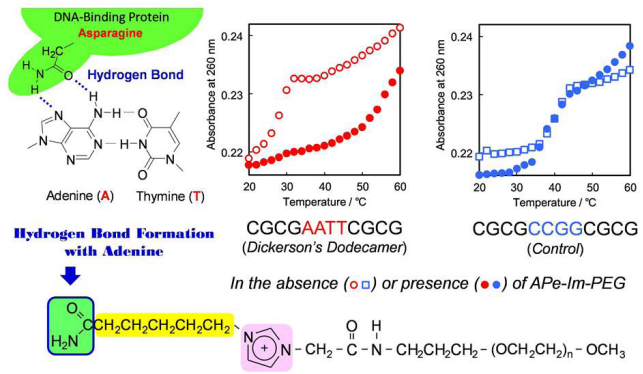

Figure 1. Mono-ion complex (MIC) between pDNA and ω-amide-pentylimidazolium end-modified PEG (APe-Im-PEG).

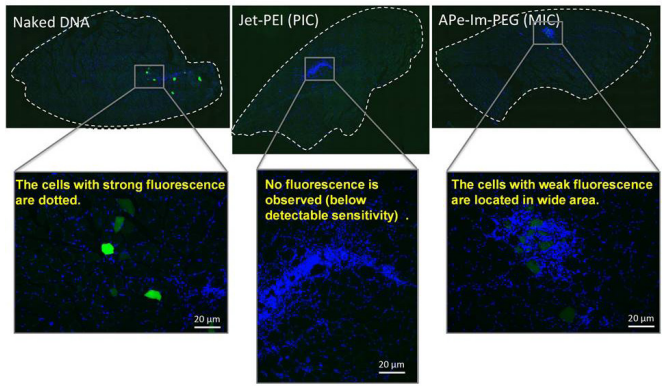

Figure 2. EGFP expression in skeletal muscle by the MIC between pDNA and APe-Im-PEG.

926. In Vivo Engraftment of T Cells Transfected Using Soluporeis Superior Compared with Electroporation-Based Systems

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Solupore® is a vector-free intracellular delivery platform that enables development and manufacture of cell therapies. Membrane-disruption-based methods, such as Solupore®, that enable intracellular delivery of various cargo types for clinical applications have been proposed as attractive candidates as next-generation delivery modalities because of potential benefits for safety, regulation and production [1]. Electroporation is the most widely used such method, including electroporation-based methods such as nucleofection, but disadvantages include toxicity and proliferation stalling. Solupore® uses reversible permeabilization to achieve rapid intracellular delivery of cargos with varying compositions, properties and sizes [2]. A permeabilizing delivery solution containing a low level of ethanol is used as the permeabilizing agent. The technology achieves intracellular delivery and subsequent reversal of cell permeabilization by precisely controlling the contact of the target cells with this solution. The process is rapid and cargo transfers directly into the cytoplasm by diffusion in an endocytic-independent manner. We have termed the method 'soluporation'. Here we compared the phenotype and functionality of primary human T cells following soluporation (Solupore®), nucleofection (4D-Nucleofector™) and electroporation (Neon®). GFP mRNA or 3kDa Dextran were delivered to T cells or PBMC. The extent to which the transfection systems perturb T cells was investigated by examining gene and protein expression using Affymetrix transcriptome-wide gene-level expression profiling microarrays and flow cytometry respectively. Effects on cell functionality were investigated by measuring cell proliferation and IFNγ production *in vitro* while their ability to engraft *in vivo* was evaluated in a humanised NSG mouse model. Following GFP mRNA delivery, GFP expression at 24 hr in soluporated and nucleofected cells was >80% while expression in electroporated cells was on average 60%. Gene expression analysis of 20,893 mRNAs at 24 hr post-transfection identified 317 genes differentially expressed in electroporated cells compared with 32 and 24 genes for soluporation and nucleofection respectively which were both equivalent to background level of false positives. Protein expression of T cell surface markers CD3, CD4 and CD8 was unchanged in soluporated and nucleofected cells at 24 hr post-transfection whereas expression of CD4 and CD8 was reduced in electroporated cells. Proliferation of soluporated cells over 7 days following transfection was similar to untreated controls while proliferation of nucleofected and electroporated cells was reduced. IFNγ secretion by all groups was similar to untreated cells. Soluporation did not affect the capacity of human $CD45^+$, $CD4^+$ and $CD8^+$ cells to engraft in the spleen of NSG mice up to 28 days post-infusion. In contrast, nucleofection significantly reduced engraftment levels in both the blood and spleen. This study demonstrates that Solupore® does not perturb gene expression or cell surface markers in T cells, unlike electroporation. Furthermore, cell proliferation and *in vivo* engraftment is superior in soluporated cells compared with nucleofected cells. Thus the Solupore® technology is gentle yet highly reproducible, automated, and scalable and has the potential to enable a broad range of T cell engineering applications. References 1. Stewart et al. *In vitro* and *ex vivo* strategies for intracellular delivery. Nature. 2016. 538(7624):183-192. 2. O'Dea et al. Vector-free Intracellular Delivery by Reversible Permeabilization. PLOS ONE. 2017. 12(3):e0174779.

927. Incorporating CCL2-GPI into Gesicles to Enhance the Efficacy of CRISPR/Cas9 for Inactivating the HIV Provirus

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Strategies to increase the efficiency, target cell specificity, and temporal control of the CRISPR/Cas9 gene editing system is paramount for its application to human therapies. Utilizing a specific microvesicle termed a "gesicle", we can deliver Cas9 in a ribonucleoprotein form, resulting in transient Cas9 activity. We show that gesicle-mediated delivery of CRISPR/Cas9 can disrupt HIV proviral activity in HIV-NanoLuc CHME-5 E9 cells when coupled with guide RNA's targeted to the HIV long terminal repeat. These cells contain a non-replicative HIV provirus which is modified to co-express NanoLuciferase to report proviral activity. To further enhance the specificity and efficacy of gesicle-based transduction into our cells of interest, we altered the membrane components of the gesicle producer cells (HEK293FT) to co-express a membrane-tethered version of the chemokine CCL2. CCL2 is a well-established migratory factor for monocytes, macrophages, and microglia which express the cognate receptor CCR2. We show that transfection of HEK293FT producer cells with the GPI-(Myc)linker-CCL2 construct results in the expression of (Myc)linker and CCL2 based on immunocytochemistry and western blot analysis. Gesicles were then prepared with and without the co-expression of tethered CCL2 to produce CCL2-GPI gesicles or control gesicles respectively. Both gesicle populations contain Cas9 and LTR-targeted gRNAs, and were applied to HIV-NanoLuc CHME-5 E9 cells. The CCL2-GPI-containing gesicles significantly decreased proviral activity both basally and after TNF-α stimulation when compared to control gesicles. These data suggest that membrane bound chemokines may serve as a strategy to enhance the efficacy of gesicle-mediated delivery of CRISPR/Cas9.

928. Delivery of Artificial Transcription Factors as an Approach to Prenatal Treatment

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There are several genetic disorders that can be diagnosed prenatally which have the potential to be treated in-utero. Even with the array of new tools available, delivery to the developing embryo in a treatment context is a challenge. One method of treatment for such diseases could be to utilize a cell penetrating peptide delivery approach to cross the placental barrier. In this study, we report that the placental barrier can be crossed by a set of domains attached to the third homeodomain of HIV- commonly referred to as TAT. This construct was fused to a zinc finger-based Artificial Transcription Factor (ATF) that was engineered

to treat Angelman Syndrome as a systemic injectable. Pregnant mice were injected with this purified ATF protein for a varying number of days prior to giving birth. Subsequent staining for immunologic tags and protein markers demonstrated the successful delivery of the protein construct as compared to control litters. Immunohistochemistry of pups whose mothers received five injections before giving birth revealed widespread distribution of the ATF throughout the body, indicating that this type of protein-based delivery has the ability to cross the placental barrier.

929. Mesenchymal Stem Cell-Derived Extracellular Vesicles as Delivery Agents for miRNAs

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Mesenchymal Stem Cells (MSCs) are non-hematopoietic multipotent stem cells that play an important role in wound healing. MSCs also have the proven ability to migrate to the sites of tumors and metastases, raising their potential as tumor-targeted delivery vehicles. MSCs secrete a range of factors including extracellular vesicles (EVs) which contain genetic information including microRNAs. EV-encapsulated microRNAs (EV-miRs) are then taken up by recipient cells, and this natural exchange of genetic information can be maipulated. This group recently reported engineering of MSCs to secrete a tumor suppressor microRNA, miR-379. The released EVs enriched with miR-379 were shown to reduce breast cancer growth In Vivo. The data suggested that MSC-secreted EVs may represent a novel and effective way to treat metastatic breast cancer. However, there a remains a lack of knowledge regarding regulation of EV-miR content, release and uptake by recipeint cells. Aim: This study aimed to further investigate the miRNA content of MSC-secreted EVs, and the potential to manipulate it. Methods: Bone-marrow derived MSCs were isolated from the iliac crest of healthy volunteers and cultured. Cells were transduced with lentivirus to express elevated miR-379, miR-504 or a control sequence (NTC). MSC-secreted EVs were isolated by differential centrifugation, microfiltration and ultracentrifugation. The morphology, size and number of isolated EVs was characterized using Transmission Electron Microscopy, Western Blot and Nanoparticle Tracking Analysis(NTA) respectively. EV-miRs were analysed by microRNA array and RQ-PCR. Results: MSC-secreted EVs were successfully isolated and confirmed to have the correct morphology and size (30-120nm), and to express exosome-associated proteins. Although protein yield has been frequently been used in the past as a surrogate indicator of EV quantity, no relationship between the number of EVs measured using NTA, and protein yield was detected. Array analysis identified >400 miRNAs encapsulated in MSC-secreted EVs from a panel of 2000 analysed. The panel detected remained remarkably stable across cell passage. Lentiviral transduction of MSCs resulted in a significant increase in miR-379 and miR-504 expression in the cells. In the case of miR-379, this was also reflected in secreted EVs, which contained elevated miR-379. In contrast, despite a significant increase in cellular expression of miR-504, the level in secreted EVs remained unchanged. The data presented reveals a range of EV-miRs secreted by MSCs, and highlights selective packaging of cellular miRNAs into EVs. Factors mediating the selective miRNA packaging and subsequent release remain to be elucidated. Understanding the elements governing MSC-EV content and migratory itinerary will be critical to advance their exciting potential in the therapeutic setting.

930. Gesicles: A Promising Nucleic Acid Delivery Tool

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Gesicles are a promising tool to deliver DNA and RNA into primary cells. Gesicles are small cellular vesicles containing, across their membrane, the envelope glycoprotein of the vesicular stomatitis virus (VSV-G). In presence of polybrene, Gesicles were able to deliver plasmids in several animal cells. Unfortunately, their production, characterization and use for nucleic acid delivery are poorly documented. First, best parameters to transfect the producer cells for VSV-G expression were identified in static culture. Then, a time course of Gesicles production was assessed. Interestingly, we demonstrated that specific mutations in the VSV-G sequence allow a Gesicle production. Transmission electron microscopy of Gesicles revealed spherical particles with membrane mimicking enveloped viruses. Immuno-gold staining confirmed that Gesicles contain VSV-G. Dynamic light scattering analysis reported that Gesicle production have a mean size of 140 nm in a PBS solution. Gesicle proteomic analysis identified 877 human proteins originating from the producer cells. These proteins are mainly represented by enzymes (52%), cytoskeleton (19%), receptors (15%) and ribosomal (12%). The analysis also identified 692 bovine proteins from the serum used during cell culture process. Then, parameters involved in the DNA-Gesicles delivery such as component concentrations, incubation time, assembling order, volume and nature of solutions were studied to determine the simplest transfection method. Stability assays also demonstrated robustness of Gesicles to several freezing and thawing cycles and long term storage at +4°C, -20°C and -80°C. Finally, transfections experiments delivered plasmids in 70% of HEK293 cells, 55% of HeLa cells and 22% of human myoblasts without cytotoxicity. Gesicles also delivered large plasmids. Furthermore, siRNA-induced gene silencing was successfully achieved by a 58%-inhibition of over-expressed reporter gene. In conclusion, Gesicles are a promising tool for nucleic acid delivery under further optimization which could be useful for several applications oriented toward cell and gene therapies.

931. Clinical Scale Production of Exosomes, a Novel Therapeutic Platform for the Delivery of Biomolecules

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Exosomes are vesicles comprised of lipids, proteins, carbohydrates, and nucleic acids that are produced by cells. These complex nanoparticles transit the body and are taken up by cells, thereby altering the recipient cell biology. We seek to harness the therapeutic potential of exosomes by loading pharmacological biomolecules into exosomes to co-opt the natural trafficking and uptake of exosomes. Current state of the art technology for purifying exosomes requires unit operations such as gradient ultracentrifugation that do not have a clear path to scalability in manufacturing. We have developed an exosome purification process that utilizes unit operations that are rapidly scalable from bench to pilot to clinical scale production in a GMP environment. Here we present data from our proprietary manufacturing process at the clinical scale. We show that process performance, product quality, and product yield are consistent from the bench scale to the clinical scale. The ability to produce exosomes at a clinical scale removes a significant bottleneck in the development of exosomes for therapeutics.

Vector and Cell Engineering, Production or Manufacturing III

932. RNA-Guided CFTR Activation by CRISPR/Cas9 Complexes in Airway Epithelial Basal Cells and Differentiated Air-Liquid Interface Cultures

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Airway basal stem cells (SC) are a therapeutic target for chronic airway diseases such as cystic fibrosis since they are considered the professional SC of the human airways. Additionally, the ability to expand airway basal SCs in conditionally reprogrammed cultures (CRC) has provided new tools personalized medicine directed at identifying of small molecule corrector and potentiators for specific CFTR mutations. However, CFTR expression often declines with passage under CRC conditions. We hypothesized that this limitation could be effectively overcome through transcriptional activation of CFTR using a nuclease dead version of CRISPR/Cas9 that anchor a dCas9 transcription activator fusion proteins to the CFTR promoter. Here we show that the dCas9-VP64-p65 system, together with gRNA targeting the CFTR promoter, can significantly activate CFTR gene transcription in primary basal cells. Mouse tracheal basal cells were cultured in SAGM under fibroblast-free conditions in the presence of Rho-kinase inhibitor. Cells were transduced with lentiviruses harboring dCas9-VP64 and MS2-p65-HSF1, then selected to obtain polyclonal stably integrated pools. Guide RNAs were designed to target the first 500 bp upstream of CFTR gene transcriptional start site and three guides with the best off-target scores were selected. Guide RNAs were transfected into dCas9-VP64 with or without MS2-p65-HSF1 expressing mouse basal cells and TaqMan qPCR for CFTR mRNA was performed at 48 hours following transfection. The fold-increase in CFTR mRNA expression was normalized to β-actin expression by the $\Delta\Delta C_T$ method. Transfection of gRNA1, gRNA2 or gRNA3 yielded significant activation of CFTR mRNA expression in dCas9-VP64 expressing mouse basal cells (22+/-3.4-fold with 20pM gRNA; 142+/-61-fold with 100pM gRNA). Fold inductions were additive with the three gRNAs together (41+/-17.4-fold/20pM gRNA; 386+/-39.4-fold/100pM gRNA). dCas9-vp64 paired with MS2-p65-HSF1 provided higher transcription activation for CFTR gene than dCas9-vp64 alone. We found dCas9-VP64-p65 system drove 1311+/-254.1-fold CFTR mRNA activation in gRNA combination group. Differentiation of gRNA transfected dCas9-VP64-p65 expressing mouse basal cells significantly stimulated CFTR-dependent chloride currents ~2.5-fold over mock-transfected controls. In summary, we have developed an efficient method of augmenting CFTR gene expression in primary basal cells that could improve the utility of CRC differentiated airway epithelia for identify small molecules therapeutics for specific CFTR mutations. This approach is also currently being applied to both human and ferret airway basal cells.

933. Exploiting AAV Receptors in the Production Biology of rAAV

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Recombinant adeno-associated virus (rAAV) is a safe and efficient means of potential treatment for a variety of genetic diseases. Despite advances in vector engineering and optimization of rAAV production protocols, achieving high-titer rAAV from preferred cell lines remains a critical challenge to provide effective dosing at reasonable cost. A deeper understanding of rAAV biology may be required to better adapt production processes. A conventional method for rAAV production is called 293-triple transfection by which plasmid DNAs carrying a vector genome, AAV rep/cap and adenovirus helper genes are co-transfected into HEK 293 cells. Roles for each of these elements within rAAV production biology have been extensively investigated. Interestingly, distribution profiles of packaged rAAVs in cellular compartment and culture medium are variable among different serotypes of AAV capsids. Generally, rAAV2 is a poor producer with a majority of packaged virions retained in cells whereas other high producer serotypes such as rAAV8 have substantial viral release into culture medium. While it is presumed that these rAAVs in the media may "re-infect" producer cells, little is known of this phenomenon or how it may influence rAAV production efficiency. We hypothesized that exploiting AAV receptors in rAAV production biology may improve productivity of poor producers, possibly enabling the development of a "continuous production and harvesting" platform. To this end, we generated two modified 293 cell lines with two genes encoding known AAV receptors: *KIAA0319L* (AAVR) and *SDC2/HSPG* (Heparin Sulfate Proteoglycan) knocked out

(KO) by using CRISPR/Cas9. Following validation of KO genotypes, we found *SDC2* KO had little measurable impact on transduction of 293 cells by rAAV2, indicating alternative cell surface receptors may be responsible for initiation of infection. However, infection of *AAVR* KO cell line displayed drastically reduced transduction, confirming functional receptor depletion. The packaging ability of each KO line was assayed via qPCR of packaged viral genomes in cell pellets and culture media after triple-transfection. While distribution of rAAV packaged by *AAVR* KO cells appears to favor cellular distribution, the *SDC2* KO line has little impact on viral distribution and total titers. More thorough comparison of WT and 293 *AAVR* KO cell lines was carried out by full-scale production and purification of an AAV2 vector. We found packaged vector genome titers are $9.99x10^{11}$ and $3.44x10^{12}$ gc/mL for *AAVR* KO and WT HEK 293 cells, respectively, representing ~70% reduction for the *AAVR* KO. Taken together, our data supports a model of rAAV production where re-infection is not required, but appears highly favorable for enhanced titer. In light of these findings, experiments to activate or augment producer cell viral infectivity by further exploitation of AAV receptor biology are underway.
* These two authors contributed equally to this study.

934. Automated End-to-End Manufacturing Solutions for Car-T Immunotherapies

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Introduction: Cell therapies have been in clinical development to treat an array of human diseases. Autologous therapies, such as CAR-T cells for cancer therapy, pose a major manufacturing challenge; the solution is to have the entire process performed via a closed and automated system. The system which we used is a system specifically designed to meet these needs. It utilizes a disposable cassette, which can be tailored to perform an array of end-to-end process including T-cell activiation, viral transduction, cell expansion, as well as washing and concentration. Our work highlights the successful translation of a typical manual CAR-T process into the Cocoon system to reduce cost and maximize process efficiency and quality. **Methods:** The CAR-T process generating HER-2 reactive CAR-T cells was performed using the following critical process parameters: starting inoculation of 100 million PBMCs, CD3/CD28 activation, IL-2 and IL-7 were supplemented into T-cell growth media for culture expansion with an optimized and defined feeding strategy. The system was programed to run the entire process after inoculation automatically, without manual intervention. Single-use sensors in the disposable cassette were used to monitor temperature, pH and DO in real time. The multiple cassette chambers that are connected via fluidic channels enabled automated feeding and addition of process components. Some of the chambers are temperature controlled at 4°C for media and reagent storage, while others include elements for warming, mixing, washing and concentrating cells, allowing for a fully closed process. The in-process samples were drawn for cell counts and viability. At the end of the harvesting process, FACS analysis was performed with the following panel: CD4, CD8, NGFR, IFN-γ and TNF-α etc. **Results:** In 10-14 day cultures, the T cells reached approximately 2 Billion in the system while GFP Lentivirus was used to optimize transduction efficiency. Automated runs and associated manual controls were able to maintain both CD4+ and CD8+ T cell subsets. We also evaluated the percentage of CAR-T cells present in each population of T cell subset. Interestingly, there was a higher detection of NGFR (indicative of the CAR construct) in the CD4 fraction than in the CD8 fraction in all samples. In summary, automated CAR-T process in our system yields a healthy populations of T cell subsets. **Conclusions:** This system is a viable solution to translate labor-intensive CAR-T process into a fully automated and highly controlled system, thus allowing scalability, high yield, reduction of manufacturing cost, and gaining better process control to yield high quality CAR-T cells.

935. Production of Lentiviral Vectors Using 293T Cells Adapted to Grow in Suspension with Serum-Free Media

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Lentiviral vectors (LVs) are increasingly utilized in cell and gene therapy applications as they efficiently transduce target cells such as hematopoietic stem cells and T-cells. For clinical use, LVs have been produced from adherent HEK293T cells under current Good Manufacturing Practices (cGMP). The manufacturing of the clinical LVs in cGMP usually starts with growing HEK293T cells in cell factories and subsequent transient transfection with plasmids. These processes are time-consuming and labor intensive, particularly in the cGMP, and it is challenging to manufacture LVs in a scaled-up manner necessary to meet the clinical needs and commercialization in the near future. In addition, the use of animal-derived serum increases the risk of contamination by adventitious viruses and suitable supplies can be difficult to obtain. To overcome these shortcomings with large scale LV production, we have developed a suspension-based production scheme. First, we adapted HEK293T cells to grow in suspension using commercially available and chemically-defined serum-free media. The newly adapted cells, referred to as SJ293TS, grow at a rate comparable to adherent HEK293T cells (24-hrs doubling rate of 2.2 ± 0.16 and 2.1 ± 0.19, respectively). Next, we optimized transfection conditions in SJ293TS cells. SJ293TS cells generate LV titers equivalent to HEK293T cells, with simple GFP-reporter vectors having titers in the mid 10^8 TU/ml range. To test cell line stability, the SJ293TS cells were continuously cultured and every week or two LV was produced by transient transfection. LV production remained optimal for at least 2 months. A further improvement included replacing the β-lactamase open reading frame on helper and vector plasmids with a non-antibiotic selectable marker (RNA-OUT, Nature Technology, Lincoln, NE) resulting in a modest improvement in LV production ($3.4 x10^8$ vs $5.2 x10^8$ TU/ml, N=3). One liter batches of LV produced by SJ293TS cells processed using ion-exchange and diafiltration resulted in overall yields of $61\% \pm 14\%$ (range 45-89%, N=11) which is much higher than conventional cell factory-based HEK293T production. SJ293TS-derived LV transduce primary human CD34+ cells with similar efficiency as LV made from 293T cells when matched for MOI. The use of this new cell line will

allow for direct scaling up of LV production in GMP facilities. Future experiments are focused on demonstrating the suitability of the SJ293TS cell line in single use bioreactors at 10 to 40 liter scale.

936. Acoustic Cell Processing and Affinity Selection for CAR T-Cell Manufacturing

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Acoustic Cell Processing is a unique acousto-fluidics platform technology for shear-free manipulation of cells using ultrasonic standing waves. The platform has broad applications in the field of cell and gene therapy, e.g., cell concentration and washing, cell culturing, microcarrier/cell separation, acoustic affinity cell selection and label-free cell selection. The acoustic radiation force exerted by the ultrasonic standing wave on the suspended cells in combination with fluid drag forces and gravitational forces is used to manipulate the cells and achieve a certain cell processing unit operation, e.g., separate, concentrate and wash. The technology is single-use, continuous, and can be scaled up, down or out. It therefore allows for a flexible and modular approach that can be customized to process a desired cell count, cell culture volume or cell concentration within a given required process time.Utilizing its proprietary multi-dimensional standing wave platform, FloDesign Sonics (FD Sonics) has been developing two applications for cell and gene therapy manufacturing, an Acoustic Concentrate-Wash (ACW) and Acoustic Affinity Cell Selection (AACS) system for closed and shear free Cell and Gene Therapy manufacturing, namely CAR-T immunocellular therapies. The ACW technology has been applied to primary T-cell cultures of 1 Liter (L) with two ranges of cell concentrations, a low cell concentration of between 1 to 2 million cells per milliliter (ml), and a second range with cell concentrations ranging from 30 to 40 million cells per ml. Thus, the number of total viable CAR-T cells processed ranges from 1 to 40 billion. The process flow rate varies from 2-5 L/hour with average cell recoveries of more than 80% in less than 1 hour. At low cell concentrations, a 150-fold volume reduction has been achieved with cell concentrations in excess of 250 million cells per ml. At high cell concentrations, a 20-fold volume reduction and more than 500 million cells per ml were measured. The efficiency of the buffer exchange process is higher than 90%. The AACS technology is a scalable acoustic affinity cell selection method using a non-paramagnetic clinical-grade affinity bead for positive or negative cell selection. A multi-dimensional acoustic standing wave is then used to separate the affinity bead-cell complexes from the unbound cells, thereby completing the process of a negative or positive cell selection. A population of 1 billion CAR-T cells containing 30% T-Cell Receptor positive (TCR+) and 70% T-cell Receptor Negative (TCR-) cells has been depleted of more than 95% of its TCR+ population. The TCR- cell recovery for this process was above 70% and the full process took less than 2 hours. ACW and AACS are powerful acoustic-based cell processing technologies that lower cost and risk while enabling a modular, automation-friendly manufacturing process for cell and gene therapy manufacturing.

937. Two-Photon Polymerized Poly(Caprolactone) as a High-Resolution, 3D-Printed Cell Delivery Scaffold

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Degradable polymer scaffolds are a promising meansproduction for use to successfully deliver cell therapies to various tissues, but three-dimensional control of their microstructure is difficult to accomplish. In this work, we demonstrate the use of acrylated poly(caprolactone) (PCL), paired with two-photon polymerization (TPP), to achieve precise architectural control of a broadly applicable biomaterial. Specifically, we optimized polymer mixture composition and TPP settings using a scaled-down tissue scaffold model. Of note, the size of resolvable features and polymerization threshold were both minimized by increasing the abundance of cross-linkable groups. We also validated the compatibility of TPP PCL scaffolds in the degenerate porcine sub-retinal space, a surgical model for one of the most sensitive tissues in the human body. No intraocular inflammation was observed one month after transplantation, indicating that the material was well-tolerated. Utilizing results from both our biocompatibility study and methodical analysis of TPP, we created high-resolution degradable cell delivery scaffolds appropriate for use in a small animal validation study. Overall, the results of these experiments highlight the promise of TPP and PCL in various cell therapy strategies and the many ways in which they could be used to improve human health.

938. Generation of Helper Virus-Free Adeno-Associated Viral Vector Packaging/Producer Cell Lines Based on a Human Suspension Cell Line

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The emerging number of clinical trials in the gene therapy field poses the challenge to the industry to produce viral vectors in a scalable, reproducible and cost-efficient manner. To address this issue, our CAP-GT platform comprises high density, serum free suspension cell lines that enable reproducible, scalable transfection and high titer production of viral vectors. A first adeno-associated virus (AAV) based vector was already approved as gene therapy product in clinical applications. Attractive features of AAV as a gene therapy vector are e.g. its lack of pathogenicity and its ability to transduce dividing and non-dividing cells. Moving away from mainly targeting ultra-rare diseases and taking more common indications into focus will need to see significant improvements concerning productivity and consistent quality of AAV vector production in order to ensure supply. For this purpose, we are developing a helper virus-free packaging cell line that can easily be turned into a producer cell line by only one additional step of cell line development. Base of this packaging cell line is the generation

of a cell line with stable Tet-inducible expression of Rep proteins. Extensive screening of Rep expressing single cell clones resulted in clonal cell lines which produced high AAV titers upon induction and transfection of the missing components. In a next step, the adenoviral helper functions E2A, E4orf6, VA RNA were introduced. Finally, the capsid function is stable integrated. Introduction of GFP as transgene flanked by the ITRs is then resulting in a proof of principle producer cell line. This approach will enable a consistent quality production of AAV vectors that abolishes the drawbacks of transient transfection concerning reproducibility, consistency and high costs for GMP-grade DNA. Process optimization in regard to process duration, feeding strategy etc. is currently ongoing to further increase the vector yields.

939. Development of Novel Inverted Quasi-Spherical Cell Culture Systems for Ex Vivo Genetically Regulated Cell Therapy

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In the field of genetically regulated cell therapy, the gene delivery efficiency is crucial contributor to any given therapy's effects. In this study, inverted quasi-spherical (iQS) ex vivo gene delivery substrates were fabricated to enhance the efficiency of viral vector delivery into targeted cells and the efficiency of transgene expression. The iQS substrates were composed of stable polydopamine (pDA) hydrophilic and nano-roughness supherhydrophobic titanium dioxide (TiO_2) layers. The sub-micron dot patterns of the exposed hydrophilic surfaces limited the contact areas of the hanging droplets, so changing the exposed hydrophilic contact areas could induce the unique curvature of droplets. The iQS substrates gave the droplets a quasi-spherical curvature and the iQS droplets' unique microenvironments provided suitable cell culture conditions because they allowed for frequent intracellular and cell-virus interactions. In this study, the iQS substrates were shown to have the potential to serve as an ex vivo platform in therapeutic systems based on human neural stem cells (hNSCs) and adeno-associated viral vectors (AAV). The iQS substrates' bioactive microenvironments were shown to have increased AAV transduction efficiency, neurosphere size, and secretion rate of therapeutic cytokine (IL10; interleukin-10). This novel iQS substrate was shown to have the potential to serve as a platform in ex vivo genetically regulated cell therapies.

940. Development of the First World Health Organisation (Who) International Lentiviral Vector Standard

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Assuring the quality and safety of lentivirus-based products presents a great challenge because they are cell-based multigene products that include viral and therapeutic proteins as well as modified cells. Changes in production sites and manufacturing processes have become more and more common, posing challenges to developers regarding reproducibility and comparability of results.This poster presents the development of the first World Health Organization International Standard for Lentivirus-based gene therapy products. The standard will be used to standardize assays which will provide a means to enable important cross-trial and cross-manufacturing comparisons to be made for emerging and existing lentiviral (LV) vector products. The standard will be expected to optimize the development of gene therapy medicinal products, which is especially important, given the usually orphan nature of the diseases to be treated, naturally hampering reproducibility and comparability of results.

941. Development and Evaluation of High Throughput Scale-Down Models for rAAV Chromatographic Separations for Application in cGMP Manufacturing

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As interest and demand for rAAV based therapeutics continues to grow at an accelerated pace, process developers are desperately in need of development methods which enable the examination of more critical process parameters with less material, and in less time. Scale down and high-throughput screening are approaches that can satisfy these demands and have shown great success for the development of other biological therapeutic processes. One of the best studied and most broadly applicable methodologies is the high-throughput screening of chromatographic separations. In this work, the high-throughput screening of rAAV purification using various resin modalities, including affinity, ion-exchange, and others, was developed. The ability to evaluate various binding, wash, elution and cleaning conditions was explored. Results from plate-based experiments were directly compared to chromatographic operations at the bench and cGMP production scales. These results indicate that plate-based high-throughput chromatographic screening of rAAV separations can provide high quantity, high quality, and most importantly scalable data using a fraction of the material and in a fraction of the time as conventional "small-scale" benchtop experiments.

942. Comparison of Bioreactors and Scale-Up of Sf9-Baculovirus Systems for rAAV Production

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A major hurdle to the future commercial success of rAAV therapeutics is the manufacturability of cGMP grade vector. The increase in cGMP vector required has placed scalability of the manufacturing process on the critical path for commercial development as processes transition from bench to large-scale manufacturing. In this work the impact of bioreactor and scale on the quantity and quality of vector produced, and on critical cell culture process parameters is investigated. Performance of the production process across bioreactor vessel types and across scales (1000x volumetric range) is compared for several serotypes. Scale effects on cell growth, metabolites, productivity, and product

quality profiles is discussed. These data demonstrate successful scale-up of the Sf9-Baculovirus system and serves to provide the fundamental understanding necessary to develop further scale-down models which will support future process optimization, robustness and characterization efforts.

943. Rapid Development and Seamless Scale-Up of Genetic Nanomedicines

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In the last five years, our focus has been to develop tools and platform technologies for rapid development and seamless scale-up of nanomedicines for clinical applications. Previously, we presented data showing development of Factor VII siRNA lipid nanoparticles (LNPs) on microfluidic-based NanoAssemblr™ platform. We optimized the formulation at the 2-10 mL scale on the NanoAssemblr™ Benchtop and scaled it up to 00 mL on the NanoAssemblr™ Blaze and then 1000 mL on the 8X Scale-up System. We demonstrated that the NanoAssemblr™ platform provides seamless scale-up and can produce large-scale volumes of lipid nanoparticles with consistent results. The genetic medicine field is quickly evolving to adopt mRNA-based therapeutics for genetic editing, immunotherapy and gene replacement therapies. In this year's annual meeting, we will be presenting the development and scale-up of luciferase mRNA LNPs on the NanoAssemblr™ platform. We will optimize the formulation on the NanoAssemblr Benchtop™ at 1-10 mL and scale it up 10X on the NanoAssemblr Blaze and then 100X on 8X Scale-up System. Particle size and polydispersity index will be determined using dynamic light scattering (DLS) technique. RiboGreen Assay will be used to determine encapsulation efficiency. UPLC methods has been developed to analyze mRNA, lipid composition and determine amine to phosphate (N/P) ratio of the formulation. We will also optimize the downstream processing using tangential flow filtration (TFF) for buffer exchange and concentration of mRNA. Finally, the particles will be injected in mice intravenously and gene expression will be determined using luciferase activity (bioluminescence) in a IVIS imaging system. In agreement to our previous findings with siRNA LNPs, these studies will demonstrate that the NanoAssemblr™ platform provides seamless scale-up and can produce large-scale volumes of mRNA LNPs with consistent results. The 8X Scale-Up system can prepare up to 25 L of product in under 4.5 hours at 96 mL/min and incorporates a disposable fluid path that eliminates the need for costly and time-consuming cleaning validation.

944. The Gap between Clinician-Led Cell Therapy Evidence and Marketed Products: a Quasi-Experimental Comparative Pilot Study of Young Venture Clinical Development

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Background: There is an enormous discrepancy between number of trials and publications for more-than-minimally manipulated (MTMM) autologous cell based therapies versus approved products, representing a significant economic investment not yet realizing value for patients. While trials led by clinicians continue to demonstrate the enormous potential benefit of cellular therapies, relatively few products have been commercialized. This research aimed to examine barriers for young firms (aged <5 years) engaged in commercial development of cell therapies. **Methods:** A quasi-experimental study examined redacted clinical regulatory documentation obtained via United States (US) *Freedom of Information Act* and available institutional documents, including those submitted to US Food and Drug Administration (FDA) as part of pre-market application or response documents. Sixteen documents from established firms and 16 from young firms (incorporated ≤5 years) were coded by categorical agency comment type, and results were compared by Mann-Whitney test. Number of publications (PubMed indexed) and registered clinical trials (ClincialTrials.gov) were examined based on keyword searching. Further, semi-empirical field data were collected via interviews with firm members and data are presented descriptively to provide supporting field evidence of impact. **Results:** A total of 26,938 cell therapy trials (8,601 active) are registered to clincialtrials.gov. Further, 42,849 cell therapy articles are indexed on PubMed, with a 10-year mean publication growth rate of 7.07% (2.35-10.95%). Oncology- and cardiology-related articles together make up more than 44% of all publications and the majority of registered trials (71.9%). Of sampled regulatory documents, issues with pre-marketing application documents most frequently included inadequate preclinical model selection, inadequate manufacturing conditions, lack of batch control, and inadequate rationale for benefit in context of care pathway were most commonly cited. Semi-empirical data revealed that lack of requirements understanding and a budget-driven waterfall approach to development among young firms often leads to additional time and prohibitive investment to complete development. **Conclusions:** The immense burden placed on small firms to complete costly testing activities and limited clarity on marketing requirements places these firms at a significant disadvantage compared to large firms. This study provides initial semi-empirical evidence that can be used to improve young firm performance and inform venture investment in these firms.

Comparison of Deficiencies Observed in Pre-marketing regulatory document by firm age

	Young Firm N = 16 (<5 y)	**Established Firm** N = 16
Inappropriate preclinical model selection	3	0
Inadequate manufacturing / batch control	8	1
Indication too broad	2	2
Inadequate risk-benefit rationale	5	2
Inappropriate bioavailability (BA)/ bioequivalence (BE)	9	0
Failure to address product-specific risks	7	4
Materials sourcing	7	2
Missing/inappropriate preclinical testing	8	0
Missing/inappropriate sterility testing	7	0
Inconsistent nomenclature	9	3
Testing does not support indications	3	2
Population not appropriate	1	1
Missing documents	5	2
Document incorrectly submitted or formatted	3	0

Figure 1. PubMed Articles Addressing Cell Therapy by Year

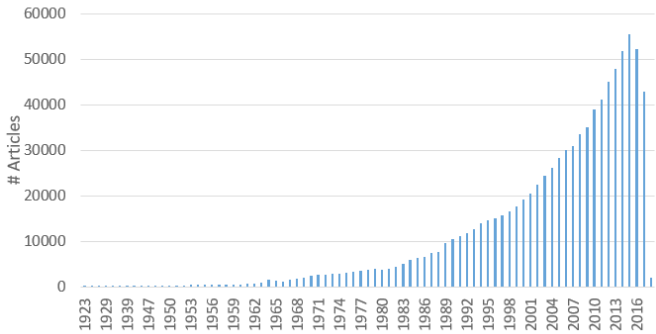
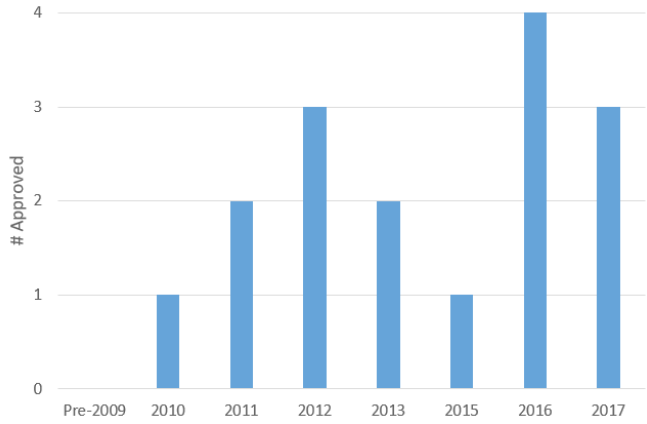


Figure 2. FDA Approvals of Gene and Cell Therapies by Year



945. AAV Titration Elisa for Standardized Gene Delivery

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A growing number of academic and industrial labs are using AAV vectors for the development of gene therapies leading to an increase in the demand for effective and reliable analytical AAV tools for R&D and manufacturing. To enable safe and effective AAV gene therapies, a dependable and reproducible quantification of accurate rAAV titers is needed to ensure safe and reliable gene transfer. Current quantification methods for rAAV vector preparations include qPCR, digital droplet PCR (ddPCR) for measuring DNA, Dot Blot and ELISA for measuring intact viral capsid protein. Key factors such as accuracy, time and cost determine which technique is ideal for standardized therapy protocols. Here we show, that a conventional sandwich ELISA currently appears to be the best format for the quantification of rAAV preparations in comparison to other quantitative techniques in terms of interassay variability and ease of use.

946. Gibco™ LV-MAX™ Lentiviral Production System for Gene Therapy Application

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Current new generation of therapies CAR - T requires the lentiviral vectors as efficient gene transfer tool to express engineered Chimeric Antigen Receptors (CAR) on the surface of the T-cells to recognize and kill the cancer cells. One problem of developing CAR T cell therapies is the high cost associated with lentiviral production. Therefore preclinical and clinical researchers have demanded their lentiviral production on a much larger scale, high-titer and in animal serum free medium. We have developed a new suspension Gibco™LV-MAX™Lentiviral production system to produce vectors in a serum free suspension platform and at high titers. This technology employs a newly developed propriety set of GMP reagents comprising of suspension cells, culture medium, supplement, transfection reagent and enhancers. With this new system we are able to produce ~1.5E+08 (TU/ml) of unconcentrated lentiviral vectors, which is at least 10 folds higher than any other published method of lentiviral production. In this report, we will describe the methods and DOE experiments that we used to identify this new suite of reagents and their application in the immune cell therapy field.

947. Aseptic Fill/Finish in a Cytocentric Isolator for Flexible Production of Cellular Therapeutics and Biologicals

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The final fill/finish step of a biologic or cell therapeutic product is a critical one for sterility. These products can't be treated harshly in a final sterilization step or the manufacturer risks severely degrading product quality. The vial, the product, the lid, and the seal all have to come together under ISO 5/ Class A conditions. Here we report media fill tests for this final step in the Xvivo System barrier isolators to test the sterility of conditions. The Xvivo GMP System is a flexible addition to an existing production line that does not utilize any room air, or require connections to external HVAC systems to provide an ISO 5 / Class A environment anywhere it is needed. We used a color-changing highly permissive microbial broth to simulate a typical cell bank fill process of 100 vials. Environmental monitoring was performed during operations, using an air sampler to draw isolator air across a contact plate. We also used contact plates to assess microbial contamination in the isolator chamber itself after use. We repeated the trial six times, incubating the vials and plates for 14 days. We found no contamination in any of the sample products or in negative controls that had not been exposed to the isolator atmosphere. We contaminated one positive control vial and one contact plate in each fill batch and each of these was positive for microbial growth. The aseptic conditions in the Xvivo System are suitable for fill/finish step and can safely give a manufacturer more flexibility in capacity and operations.

Gene Targeting and Gene Correction

948. Efficient In Vivo Selection of Gene-Targeted Hepatocytes Using Acetaminophen-Induced Liver Toxicity

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Precise gene editing by homologous recombination has low efficiency in vivo. However, selective expansion of rare gene-targeted cells is a promising approach to overcome this problem. We previously described a system for expanding gene-edited hepatocytes by rendering them genetically resistant to CEHPOBA, a compound that causes hepatotoxicity by blocking the tyrosine catabolic pathway. Here we demonstrate selection using a far more readily available and clinically applicable drug, acetaminophen. Acetaminophen-induced liver toxicity results from cytochrome P450 (Cyp)-mediated metabolism in hepatocytes, generating the toxic intermediate NAPQI. We theorized that a deficiency in the enzyme cytochrome P450 reductase (Cypor), the obligate co-factor for most Cyp enzymes would block this process and protect Cypor-deficient hepatocytes. To test this theory, a cohort of mice was administered a CRISPR plasmid targeting the mouse Cypor gene by hydrodynamic tail vein injection. Five mice from this cohort were assigned to the experimental group and were injected twice weekly with high doses of acetaminophen (400 mg/kg). Control mice (n=4) received no acetaminophen. **Results:** As expected, acetaminophen injections resulted in significant serum ALT spikes (~1000 IU/L) within 6 hours of administration. However these spikes abated after the tenth bi-weekly dose. After fifteen acetaminophen doses in the experimental group, all mice were sacrificed and liver sections were examined by Cypor immunofluorescence. Mice from the control group had ~1% Cypor-negative hepatocytes whereas experimental mice had regions of Cypor-negative staining comprising 40-51% of the liver area. Sequencing liver genomic DNA showed 35-42% Cypor-localized indels in experimental mice and ~3% in the control group. **Conclusions:** These data demonstrate robust clonal expansion of gene-modified hepatocytes using acetaminophen-induced liver toxicity. Acetaminophen-treated mice showed no significant weight loss during treatment and no mortality was observed. Clinical incidents of mild to moderate acetaminophen overdose normally resolve with complete restoration of liver function. With proper safety protocols, genetic protection from acetaminophen toxicity could be used to select gene-modified hepatocytes in humans and achieve clinically relevant levels of transgene expression.

949. Designed Zinc Finger Protein Transcription Factors for Single-Gene Regulation Throughout the Central Nervous System

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The Microtubule Associated Protein Tau (MAPT) is strongly linked to the pathogenesis of several primary tauopathy disorders, which include Alzheimer's Disease, Progressive Supranuclear Palsy, Frontotemporal Dementia, and Corticobasal Degeneration. While genetic and antisense-based approaches to lower intracellular tau levels have proven efficacious and well tolerated in the brain, the development of a single-administration tau-targeted therapy remains a long-standing goal. Advances in Zinc Finger Protein (ZFP) design and recombinant AAV capsid engineering have created new potential for the treatment of tauopathies and other diseases affecting the central nervous system (CNS). We developed ZFP repressors capable of reducing both mouse and human tau levels by up to 98% with no detectable off-target gene regulation in primary and iPS-derived neurons. When focally delivered to the hippocampus, MAPT-targeted ZFPs resulted in >90% tau reduction. Upon intravenous ZFP administration to adult mice, tau levels were reduced by 50-70% across the entire brain. Moreover, ZFP expression and tau reduction were stable out to at least six months, resulted in >80% lowering of CSF-tau, and reduced dystrophic neurites by 50% in APP/PS1 mice with advanced amyloid pathology. Transcriptome-wide specificity assessments detected no off-target activity or secondary effects of tau reduction in the hippocampus or frontal cortex 10 weeks after systemic ZFP administration. The potency, specificity, and stability of the effects that we have observed, coupled with continuing development of gene delivery to the brain and spinal cord, raise the prospect that it will soon be possible to use ZFPs to achieve permanent, CNS-wide regulation of tau or any other chosen gene.

950. Long-Term Evaluation of Genome Editing Outcomes for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a debilitating musculoskeletal disease characterized by muscle wasting, loss of ambulation, and premature death by 30 years of age. DMD is caused by deletions,

duplications, and nonsense mutations throughout the DMD gene resulting in the absence of dystrophin protein. Genome editing has emerged as a method to permanently repair the endogenous gene. Multiple groups have used AAV to deliver CRISPR/Cas9 to restore dystrophin expression and improve muscle function in mouse models of DMD. Further development of DMD CRISPR therapy requires careful preclinical evaluation of safety, host response, and the persistence of gene-edited cells. We administered AAV-CRISPR, designed to excise dystrophin exon 23 by simultaneous cutting of two gRNAs, systemically to neonatal and adult mdx mice. Excision of exon 23 will remove the premature stop codon in mdx mice and restore dystrophin expression. To comprehensively characterize gene editing outcomes, we utilized a custom unidirectional sequencing method to quantitatively determine rates of genetic deletion, inversion, AAV integration, indel formation, and chromosomal translocation at the target locus. Illumina Nextera transposase was used to fragment and tag genomic DNA, and then PCR was performed using a locus-specific forward primer and a constant reverse primer specific for the transposon DNA tag. One year after systemic administration into neonatal mice, unbiased unidirectional sequencing of genomic DNA (heart, TA, and diaphragm) showed total on-target editing (8.84%, 1.05%, 2.95%), including indel formation (2.90%, 0.52%, 0.70%), deletions (1.34%, 0.21%, 0.86%), inversions (0.52%, 0.04%, 0.56%), and AAV integrations (4.08%, 0.28%, 0.83%). AAV integrations were consistent with the canonical mechanism of AAV integration into double-strand break sites. No chromosomal translocations were detected in this experiment. To examine longevity of genome editing, we evaluated the same endpoints in mice eight weeks and one year after single-dose administration of AAV-CRISPR. Statistically significant increases in indels, inversions, and deletions, but not AAV integrations, were noted for the one-year time point in the heart, skeletal muscle, and diaphragm. RT-qPCR for edited dystrophin transcript levels shows significant increases in proportions of edited transcript and increases in overall abundance of dystrophin mRNA at one year. Dystrophin protein was detected at both 8 weeks and 1 year by western blot and immunofluorescence histology in multiple skeletal muscles and cardiac muscle. No significant differences were noted in H&E sections of skeletal or cardiac muscle in treated mice compared to age-matched untreated mdx mice. No adverse events were noted in the long-term cohort. This study establishes the feasibility of long-term genome editing in mouse models of disease indicating persistence of gene-edited cells one year after a single administration. In addition, this work shows the presence of alternative on-target genome modifications after delivery of AAV-CRISPR. Additional work will seek to characterize biological implications of these alternative on-target modifications, improve systemic gene deletion efficiency, and characterize humoral and cellular immune response with constitutive and muscle-specific promoters.

951. AAV-Vector Integration into CRISPR-Induced Double-Stranded Breaks

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Introduction: Adeno-associated virus (AAV) vectors are being used as carriers for the components of the CRISPR system or homology repair donors in genome editing applications. Here we characterized homology-independent AAV integration into CRISPR induced DNA breaks *in vitro* and *in vivo* into therapeutically relevant genes. ***Methods***: We used AAV to carry CRISPR components (SpCas9, SaCas9 and gRNA against different therapeutic targets) and transduced cells *in vitro* (U2-OS cells and primary mouse neurons). We also injected AAV vectors into hippocampi of adult and inner ears of neonatal mice. We re-analyzed the sequencing data from Bengtsson et al. (2017) to study AAV integration into the *Dmd* gene in mouse muscle. Integration efficiencies were quantified by targeted deep-sequencing and integration-specific qPCR. To allow sequencing of the entire integrated product, we created a minimal AAV construct with a 175bp long payload of the lambda bacteriophage (AAV-λ). Successful packaging of AAV-λ was validated with titration of vector genomes, capsid ELISA, and transmission electron microscopy. TA cloning was used to sequence the integration product after AAV-λ transduction of U2-OS cells co-transfected with guide RNAs and Cas9 encoding plasmids. Finally, AAV was PCR amplified from the genome after *in vitro* transduction and *in vivo* injection to reveal genome-wide random and CRISPR associated AAV integration (AAV-Seq). ***Results***: We observed 5-30% integration efficiencies as compared to insertion and deletion (indel) formation in the case of several genes (*Tmc1, APP, Mecp2, Dnmt3, FANCF, Dmd, Tor1A*) *in vitro* and *in vivo* after transduction with AAV. AAV integration was present not only in on-target, but also in validated off-target loci. Targeted deep-sequencing of PCR products revealed that one of the breakpoints in the AAV construct is found in the ITR, more specifically in the hairpin loops. AAV ITR integrants were present in flip and also flop configuration, with the 'a' region in ITR being the most abundant. Surprisingly, there was no difference in integration efficiency using a minimal AAV (175bp) compared to a full-length AAV construct (4.2kb), as assayed by integration-specific qPCR in U2-OS cells (assayed for *Tmc1, Tor1A, APP*, *VEGF*, *FANCF*) and targeted deep-sequencing. TA cloning after AAV-λ transduction revealed that one ITR is always present in the integrant sequence, while the other end is chewed back from the ITR and variable length sequences (including the full-length sequence) are integrated in CRISPR-induced breaks; with or without indels in the target genomic DNA. Finally, unbiased AAV-Seq identified genome-wide random and

CRISPR-induced on- and off-target integration of AAV into *Tmc1, APP, Mecp2 and Dnmt3b* genes. ***Conclusion***: Integration of AAV vectors is highly frequent after CRISPR delivery and it appears to be dependent on the viral ITR. AAV capture may serve as a sensitive marker for the presence of DNA breaks and can be used to describe CRISPR-induced DNA breaks in the genome. It could also potentially be applied to describe nuclease action in human clinical trials. The data generated in this study might also contribute to the development of AAV-CRISPR applications with lower off-target activity.

952. Non-Viral Delivery of ZFN mRNA Enables Highly Efficient *In Vivo* Genome Editing of Multiple Therapeutic Gene Targets

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Zinc finger nucleases (ZFNs) can be engineered to target virtually any chosen sequence in the genome. We have previously shown ZFNs can be packaged into adeno-associated viruses (AAV) and delivered intravenously into mice and non-human primates to induce highly efficient genome editing in the liver. Lipid nanoparticles (LNP) are a novel delivery vehicle that enables repeat administration and are not limited by the presence of pre-existing neutralizing antibodies in the serum of treated subjects. Here we show that LNP packaged with mRNA encoding ZFNs targeting intron1 of the murine *Albumin* locus co-delivered with AAV comprising either a promoterless human *IDS* or *FIX* transgene donor results in targeted gene insertion, and subsequent therapeutically-relevant levels of enzymatic activity (1950 nmol/hour/mL) and protein expression (1015 ng/mL), respectively, within the plasma (up to 7700-fold greater than wild type levels, and 8-fold higher than in previous mouse studies for human *IDS*). In addition, repeat administration of the mRNA-LNP after a single AAV donor dose significantly increased levels of genome editing and transgene expression (approximately double after 2-3 doses). For gene knockout applications, ZFNs were designed against the murine *TTR* coding region, a clinically-validated gene knockout/knockdown target for treatment of transthyretin-related amyloidosis. Lead state-of-the-art ZFN candidates, delivered as mRNA via electroporation, were capable of yielding >99% indels within murine liver cell lines *in vitro*. These ZFNs were then produced as mRNA, packaged into LNP, and injected intravenously into wildtype mice. We observed up to 73% indels in liver tissue and 91% protein knockdown in plasma following mRNA-LNP doses between 0.05 and 0.8 mg/kg, which was stable out to 35 days post-dosing. These results demonstrate LNP-mediated ZFN mRNA delivery can drive highly efficient levels of *in vivo* genome editing and can potentially offer a new treatment modality for a variety of diseases.

953. Improved Genome Editing through Inhibition of the FANCM Pathway

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Homologous recombination (HR)-based genome editing has not only been exploited for gene therapy applications but also to generate cell lines and animal models carrying knockouts or specific genetic modifications that have been used to study biological processes. Adeno-associated virus (AAV) has been exploited for genome editing because of its inherent ability to induce relatively high rates of HR with and without the use of nucleases. Several laboratories including ours have described nuclease-free AAV-mediated HR efficiencies of up to 1% of transduced cells. Based on the high HR efficiencies obtained with AAV, our laboratory has recently developed a promoterless nuclease-free AAV-mediated gene targeting approach (Barzel *et al*., Nature 2015). Using this method, the therapeutic gene is flanked by host genomic homology arms directing HR near the end of the selected highly transcribed gene. The resultant chimeric mRNA makes both the endogenous protein and the therapeutic gene of interest. This process has been named GeneRide^R and has been used to ameliorate the bleeding diathesis in both adult and neonatal hemophilia B mice, and three other mouse models of metabolic liver diseases. We determined that 0.4 - 1% of liver cells were genetically modified. However, a larger number of modified cells would be desirable in order to increase the therapeutic potential of GeneRide^R and other AAV-HR-based gene therapy approaches. Aiming to find ways to increase the HR efficiencies, we performed an unbiased genome screen using a library of mutagenized haploid human cells (HAP1) to identify genes that when absent, would enhance AAV-HR. To do this, the HAP1 library was transduced with a promoterless AAV targeting construct designed to target the human *Glyceraldehyde-3-Phosphate Dehydrogenase* (*GAPDH*) gene and to drive the expression of a drug resistance marker upon precise *GAPDH* locus integration. The screen was performed in two experimental duplicates and the cells were selected with two different drug concentrations for 7 days. DNA was extracted from the surviving cells and followed by deep-sequencing analysis. The *Fanconi anemia (FA), complementation group M* (*FANCM*) gene was identified in both screens. After generating independent clonal knockouts of *FANCM* in HAP1 and HeLa cells, nuclease-free AAV-HR was increased by approximately 10-fold. Moreover, knockout of another protein of the FANCM pathway - RMI1 - also increased nuclease-free AAV-HR to similar levels as seen for the FANCM knockout, supporting the association of this pathway in HR. In preliminary non-optimized experiments, knockout of *Fancm* gene in mouse liver increased nuclease-free AAV-HR by 3-fold. We also studied CRISPR/CAS9-mediated AAV-HR into the *GAPDH* locus and established that HR was increased by at least 2-fold in *FANCM* knockout cells. The *FANCM* gene is highly conserved and was categorized into the FA complementation group (FANC). FANC genes encode proteins belonging to the FA pathway, which becomes activated in response to breaks in single- and double-stranded DNA. Although mutations in many of the FA complementation groups have been associated with the FA recessive disorder, no disease has been linked to mutations in the

FANCM gene. Additionally, the germline knockout of *Fancm* gene is non-lethal in mice. Thus, strategies to temporarily inhibit the FANCM pathway have the potential to increase the efficiencies of nuclease-free as well as nuclease-mediated AAV-HR approaches for both *in vitro* and *in vivo* genome editing applications.

954. rAAV-Mediated Nuclease-Assisted Vector Integration (rAAV-NAVI) Promotes Highly Efficient and Stable Transgene Expression in Somatic Tissues

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Current methods for site-specific therapeutic transgene integration in somatic tissues fall short in achieving precision, efficiency, and long-term stability. Nuclease-assisted vector integration (NAVI) is a new and exciting technology that may be an ideal platform for fulfilling such a balance, and has been used successfully for *in vitro* gene editing. NAVI relies on non-homologous end joining pathways following dsDNA vector and target genome cleavage by engineered nucleases to achieve homology-independent targeted integration of vectors into genomic DNA. The efficiency of gene editing and flexibility in genomic target slection by this approach are relatively higher than homology-directed repair (HDR) methods, and therefore, may facilitate the modification of cells normally resistant to editing by HDR (i.e. post-mitotic cells). However, the impact and potential role of NAVI for *in vivo* therapeutic gene editing have not been extensively explored. In the present study, we have combined the NAVI approach with the high transduction capacity of rAAVs and have coined this approach rAAV-NAVI. Transgene expression cassettes were designed into rAAV9 vectors to target NAVI within the *AAVS1* locus. To improve the safety of rAAV-NAVI, we have engineered our vector to be "self-targeted" by directing the removal of inverted terminal repeat (ITR) sequences from the vector payload prior to integration. Notably, ITRs are the only remaining viral elements in rAAVs, and are known to drive non-specific vector genome integration. We found that targeted gene editing of livers by facial vein administration of rAAV-NAVI (~10^{11} GC) to neonatal CRISPR/Cas9 mice was highly efficient, as confirmed by direct sequencing of genomic DNA. Four weeks after injection, we observed up to 7% of total cells had intergrated transgene, despite the relatively low dose of vector. Strikingly, following partial hepatectomy (PHx) at 3 months, liver tissues continue to display strong transgene expression, despite near undetectable episomal rAAV9. This result suggests efficient and stable integration. In addition, preliminary observations of regenerating livers indicate that transgene expression was increased ~6-fold in edited cells following PHx. Importantly, we do not detect the incorporation of ITR sequences or evidence of whole-vector genome integration in treated samples. In summary, rAAV-NAVI represents a major advance toward potent therapeutic genome editing with high flaxibility for genomic target selection and efficiency with great potential for treatment of a wide spectrum of genetic diseases. To this end, projects are underway to explore additional safe harbor loci and other genomic targets for application to clinically relevant animal models, taking advantage of the unique features offered by NAVI.

955. Direct In Vivo Transduction of Mobilized CD34 HSPCs with Adenoviral Vectors in Non-Human Primates

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Gene therapy approaches using *ex vivo* cultured hematopoietic stem and progenitor cells (HSPC) have demonstrated the potential to effectively treat a range of diseases. However, bone marrow transplantation approaches are limited by several factors. These include effectively isolating target cell populations, potential differentiation of HSPCs while in culture, and extremely high cost and associated risk of transplantation protocols. Further, physical *ex vivo* gene-editing methods such as transduction or electroporation could result in reduced cell viability or differentiation potential. This can result in a lower long-term efficacy in recipients, especially in disease settings where the above complications can be compounded. Additionally, the high cost of cells, gene editing reagents, and access to an accredited facility reduces the accessibility of *ex vivo* gene therapies to many patients suffering from blood disorders. As an alternative to *ex vivo* protocols, we aim to develop a streamlined, and direct *in vivo* injection approach for gene therapy administration. We utilized a well characterized non-human primate model of gene therapy in combination with helper-dependent Adenoviral vectors (HDAd5/35) to target HSPC populations *in vivo* via intravenous vector infusion. In order to maximize the interaction of injected vector with HSPCs, we incorporated various mobilization regimens to localize these cells into the blood. We tested 4-day administration of G-CSF and SCF either with or without Plerixafor (AMD3100) in pigtail macaques. Following intravenous injection of an HDAd5/35 vector expressing GFP, over 3% of peripheral CD34s were successfully transduced in animals mobilized with only G-CSF and SCF. Analysis of bone marrow 3 days post-injection showed 0.3% GFP positive CD34s, indicating these cells are capable of homing back to the marrow niche. Plerixafor greatly increased the frequency of HSPCs in the peripheral blood (2-4 fold) compared to growth factors alone. Following injection of HDAd-GFP, again 3% of CD34s isolated from peripheral blood expressed GFP. Accounting for the frequency of HSPCs in the blood, this results in over 11,000 transduced CD34 cells/mL. Here we document successful *in vivo* transduction of HSPCs in a clinically relevant large animal model with transduced cells relocalizing back to the bone marrow while still maintaining differentiation potential. By using a validated drug-resistance gene for *in vivo* selection of modified cells previously used by our lab, it may be possible to further enrich for transduced HSPCs and their progeny. Alternatively, this protocol could be ideal for delivering temporary gene expression of a DNA editing enzyme such as CRISPRs for targeted gene disruption, or

combined with a transposon system for permanent gene integration. Protocols like this which transition gene therapy into a syringe promise to dramatically increase the availability and safety of gene therapy treatments for patient populations worldwide.

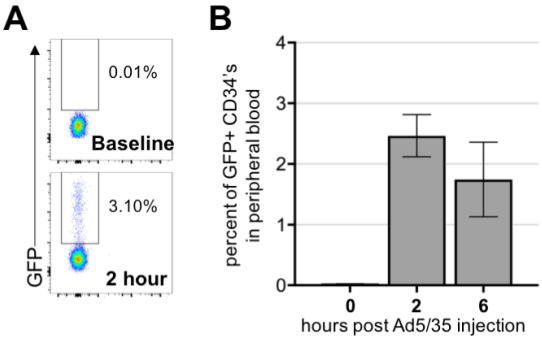

Figure 1: (**A**) Representative flow plot of peripheral blood CD34⁺ cells expressing GFP before, and 2 hours after Ad5/35 injection. (**B**) Average percent of CD34⁺ cells expressing GFP over time when isolated from blood at indicated times after Ad5/35 injection.

AAV Vectorology

956. Structure-Function Characterization of Non-Primate AAV Capsids for Their Usage as Therapeutic Gene Delivery Vectors

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All adeno-associated virus (AAV) vectors currently used in gene therapy trials or approved human gene therapy biologics are based on primate AAVs. One hurdle for AAV vectors in human gene therapy are pre-existing neutralizing antibodies (NAb) that target virus capsids, leading to vector inactivation and to a loss of treatment efficacy. To overcome this issue the capsid surface of these AAVs can be mutated to escape from Nabs. An alternative to this strategy is the utilization of AAVs that do not exist in the primate population, but have the ability to infect mammalian cells and exhibit low or no reactivity with human sera. Here we present the capsid structures of bovine AAV (BAAV), BatAAV and snake AAV (SAAV) determined by cryo-electron microscopy to a resolution of 2.57 Å, 2.98 Å, and 3.15 Å, respectively. These viruses show very low capsid sequence identities (~51-59%) to AAV2, to other primate AAVs, and to each other, as has been reported for the sequence and structurally diverse AAV4 and AAV5 serotypes. While the core structure of the capsids of these non-primate AAVs are conserved, their surface loops display unique structural features different to the primate AAVs and each other. These differences are located in the previously defined capsid variable regions (VR). Significant structural differences include a large insertion in VR-V of BAAV, large insertions in VR-III and VR-VII of BatAAV, and distinct truncations of VR-IV of BatAAV and SAAV. Many of the surface loops have previously been identified as binding sites for NAbs in the primate AAVs. Native immuno-dot-blots confirm the lack of antigenic reactivity of BAAV, BatAAV, and SAAV towards different anti-AAV antibodies or human sera. Similar to the primate AAVs, vectors packaging a recombinant genome into the non-primate AAVs can be generated by triple transfection of HEK 293 cells. While the transduction of different mammalian cell lines by BAAV vectors have previously been reported, including the identification of sialic acid-carrying gangliosides and the Chitotriose trisaccharide as receptor, nothing is known about the receptor or tropism of BatAAV or SAAV. Here we pinpoint the region of BAAV required for receptor attachment and show that SAAV also requires terminal sialic acids for cell attachment. Furthermore, SAAV-GFP vectors are capable of transducing CHO Pro5 cells leading to cellular GFP expression. This structure-function characterization of the non-primate AAVs will be beneficial to further expand the current repertoire of AAV vectors in human gene therapy applications.

957. Post-Transcriptional Fine-Tuning of AAV Vector Gene Expression for Hemophilia A Gene Therapy

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As partners in the European research network SMART-HaemoCare, we aim to develop an innovative gene therapy for the blood clotting disorder hemophilia A based on recombinant Adeno-associated viruses (rAAV). In detail, we plan to minimize the risk of bleeding in patients by expressing single domain antibody fragments (nanobodies) from liver-specific AAV vectors, in order to target and inhibit natural anticoagulants. Of note, rAAV are currently used in many clinical trials and have already been commercialized, following their emergence as a highly promising vector for gene therapy over the last five decades. Still, it is essential to further maximize their safety in patients, in particular by restricting *in vivo* vector gene expression exclusively to the desired on-target tissue. One strategy to hone target selectivity is to exploit tissue- or cell-specific expression patterns of mi(cro)RNAs. Previous studies showed that the incorporation of miRNA binding sites in the 3'UTR of a transgene can induce mRNA destabilization, in turn leading to a decrease of transgene expression in a cell-/tissue-specific manner (switch-OFF strategy). Moreover, miRNAs can be used to downregulate a bacterial repressor protein that binds to an operator sequence in the transgene promoter, leading to preferred promoter activation and transgene expression in the targeted tissue (switch-ON strategy). Here, we present a new AAV vector toolbox that significantly extends these previous systems, by combining OFF- and ON-switches in a single rAAV. To characterize our vectors, we first assessed the tissue-specific expression of selected miRNAs in a comprehensive biodistribution study in adult mice using qRT-PCR. We then validated the switch-OFF

system by injecting rAAV8 vectors carrying a luciferase reporter tagged with different permutations of up to 10 multiplexed miRNA binding sites. To achieve hepatocyte-specific expression of the nanobodies for hemophilia A treatment, we are currently customizing this switch-OFF vector to carry multiplexed miRNA binding sites for de-targeting from numerous cells/tissues including skeletal muscle and heart. In parallel, we optimized the switch-ON system by comparing various repressor systems (TetR, VanR, and E-system) *in vitro* and by evaluating the ideal number and position of operator sequences for each system. First results obtained in the context of our AAV vector toolbox show a superiority of the vanillic acid-responsive transcriptional repressor VanR. Pending validation, the best combination will be tagged with tissue-specific miRNA binding sites and tested *in vivo* in mice. Further optimizations for the switch-ON and OFF systems include the use of imperfect miRNA binding sites to fine-regulate transgene expression in target cells expressing these miRNAs. Eventually, this work will yield a robust switch-OFF/ON system for tunable transgene expression in hepatocytes with little to no activity in other organs, which will not only be pivotal for hemophilia A treatment, but for numerous other human diseases requiring efficient and selective on-target gene expression in a given tissue.

958. Wild-Type and Recombinant AAV Mitochondrial Integration and Trafficking

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In the Glybera study, recombinant adeno associated viruses (rAAV) were found to integrate within the mitochondrial genome of human and mouse skeletal muscle after rAAV1 intramuscular administration, but not upon intravenous administration. This might suggest that the occurrence of rAAV mitochondrial integration requires high mitochondrial contents and a certain transduction efficiency. Nonetheless, this phenomenon has not been further explored. To investigate whether rAAV mitochondrial integration occurs in other mitochondria-rich cell types, we studied wild-type (wt) and rAAV2 integration profiles in immortalized human cardiomyocytes. LAM-PCR yielded 1,254,997 sequencing reads enabling the identification of 14,048 and 2,396 IS from wtAAV- and rAAV-infected cells, respectively. Common integration sites (CIS) analysis revealed 1,537 CIS upon wtAAV infection and 100 CIS for rAAV-infected cardiomyocytes. OR4F29 and PCBD2 genes, both located within nuclear mitochondrial DNA segments (NUMTs), figured among the wt- and rAAV CIS retrieved. Notably, these loci were also targeted by intramuscularly-delivered rAAV1 in patients. As the NUMT are present in both nuclear (nDNA) and mitochondrial DNA (mtDNA), we further investigated the location of these IS and found that 10/10 of the rAAV IS and 57/69 for the wtAAV exhibited a higher homology with the mtDNA. For the wtAAV, 2/69 IS rather aligned to the nDNA and 10/69 exhibited comparable homology to both genomes. Therefore, our data show that both wt- and rAAV2 are able to integrate within the mitochondrial genome. To reproduce the *in vivo* findings and to further investigate rAAV integration into this organelle, we infected human primary skeletal muscle cells for the subsequent isolation of the n- and mtDNA followed by LAM-PCR. We successfully amplified rAAV junctions in both fractions and the ongoing analysis of the LAM-PCR amplicons will enable to unequivocally locate the rAAV integration events occurring within NUMTs. Complementary, we also developed a quantitative PCR-based assay to determine the rAAV integration frequency within the mtDNA. Besides, we are currently investigating the cellular mechnaisms involved in rAAV mitochondrial trafficking. As the mitochondria associated membranes (MAM) constitute contact points between the endoplasmic reticulum and the mitochondria, we hypothesized an eventual role for these cellular junctions in rAAV mitochondrial entry. Stably transfected cell lines were generated by infection of HEK293T with lentiviruses overexpressing proteins known to affect the number of MAM (cyclophillin D, glucose-regulated protein 75 and mitofusin-2). Both bulk-infected and single-cell-derived clones, as well as control cells, were later infected with a GFP-expressing rAAV2 for the subsequent isolation of both n- and mtDNA. Both fractions yielded rAAV-derived LAM-PCR amplicons and the in process sequencing data analysis will allow determining if alterations in the number of MAM affect rAAV mitochondrial integration frequency. Therefore, this study addressing rAAV mitochondrial trafficking and integration will be valuable to finer evaluate the risk of gene therapy approaches targeting mitochondria-rich tissues, as well as to assess rAAV´s potential for the treatment of mitochondrial disorders.

959. A Novel rAAV-amiRNA Platform Enables Potent *In Vivo* Gene Silencing and a Ten-Fold Enhancement of On-Target Specificity over Conventional shRNA Vectors

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Efficient and specific gene silencing are primary goals for rAAV-mediated RNAi therapeutics. High levels of AAV-delivered shRNAs can perturb the endogenous RNAi machinery, leading to cellular toxicity. Off-target activity poses a second hurdle for RNAi applications, which can complicate data interpretation in functional studies and raises safety concerns for RNAi-based therapies. Beyond these shRNA-specific challenges, our previous work has shown that shRNA-encoded sequences can redirect rAAV viral genome replication, generating truncated rAAV genomes that are functionally inert. The discovery of undesirable truncations, which are caused by hairpin sequences in rAAV genomes, highlights the importance of developing AAV-compatible gene silencing vectors that produce a high proportion of intact genomes and functional transgenes. We therefore asked whether natural miRNA scaffolds with inherently lowered structural thermostabilities could, 1) improve rAAV genome integrity, 2) be less inhibitory to cellular RNAi machinery, and 3) reduce off-target activity - all without compromising efficacy. Among 15 different mouse primary miRNAs tested by packaging into AAV vectors, the use of

a pri-miR-33 scaffold resulted in the least abundance of truncated genomes. Importantly, we show with three different gene targets (*Apob1*, *PC-1*, and hSOD1) that the *in vitro* and *in vivo* gene silencing efficacies of miRNA-33-based artificial miRNAs (amiRNAs) are as potent as Pol III driven shRNAs. Next, we profiled the transcriptomes of livers obtained from mice receiving AAV-shPC-1 or AAV-miR-33PC-1. These data revealed that both conventional shRNAs and miR-33 scaffolds promoted efficient loading of their guide strand into Argonaute. Importantly, small RNA profiling of livers treated with AAV-shPC-1 detected 13 dysregulated miRNAs (>2-fold change), while livers receiving AAV-miR-33-PC-1 did not show significant changes in the expression of any endogenous miRNAs. As a consequence, we found that the AAV-shPC-1 vector perturbed expression of 2,547 encoding genes (1,221 decreased and 1,326 increased), while AAV-shPC-1miR-33 affected only 218 genes (94 decreased and 124 increased). This work demonstrates that engineering rAAVs using the miR-33 scaffold leads to better on-target specificity and potent silencing - improving the utility of rAAVs and other viral vectors for research and clinical applications.

960. Cell-Specific Responses Following Intravenous Administration of AAV Vectors: Liver Single Cell Transcriptome Analysis of Cell Type-Specific Transgene Expression and Cell Type-Specific Vector-Mediated Transcriptome Dysregulation

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Although the liver tropism of adeno-associated virus (AAV) vectors following intravenous administration is well known, there is little data relating to the resulting liver cell type-specific transgene expression or cell-specific AAV vector-mediated dysregulation of cell biology. In this study, we have leveraged the power of single-cell RNA-sequencing to characterize the cell type-specific transgene expression as well as cell-specific vector-mediated transcriptome dysregulation in the mouse liver following intravenous administration of AAVrh.10 vectors. C57BL/6 mice were randomly divided into 3 groups (each n=3) and intravenously injected with PBS, AAVrh.10Null and AAVrh.10mCherry (10^{11} genome copies/mouse). Two weeks after vector administration, livers were harvested, dissociated into single cells, and Drop-seq performed to obtain transcriptomic profiles of single liver cells. Overall, a total of 46,500 cells were sequenced. The single-cell transcriptomic profiles of the control (PBS) mice were grouped into 3 separate clusters of hepatocytes (hep1, hep2, hep3), endothelial cells, Kupffer cells and lymphocytes. Hep1 cells, represented 85-90% of total hepatocytes (markers Serpina1a, Ass1, Car3); hep2 cells 5-10% (markers Mlxipl, Jun, Malat1); and hep3 cells 5-7% (markers Pigr, C4b, and Malat1). In the AAVrh.10mCherry-treated mouse livers, 96% of hepatocytes expressed mCherry. The % hep1, hep2, and hep3 cells expressing mCherry were similar, but the levels differed, with the level of mCherry expressed in hep1 > hep2, hep3. Interestingly, an average of 88% of other cell types also expressed mCherry, including endothelial cells, Kupffer cells and lymphocytes, i.e., it is not only the hepatocytes that are genetically modified to express the transgene. Importantly, not only did intravenous administration of the AAV vector mediate transgene expression, but also resulted in cell-specific changes in liver cell biology. In this regard, AAVrh.10Null administration resulted in 172 genes up- and 377 genes down-regulated compared to the PBS group. Among these genes, 228 genes were cell type-specific differentially expressed genes. In summary, single cell transcriptome analysis uncovers a striking heterogeneity in the liver cell-specific responses following intravenous administration of AAV vectors, including: (1) heterogeneity in expression in hepatocyte subtypes and expression in liver cells other than hepatocytes, including components of the immune system; and (2) significant cell-specific transcriptome dysregulation, with large numbers of cell-specific genes up- and down-regulated in response to AAV-mediated transduction. The data provides insights into the cell-specific consequences of AAV-mediated liver gene transfer, far beyond the well-known organ-specific expression of the vector-expressed transgene.

961. Hypothermia Improves AAV Entry into CNS across BBB and Enhances ALS Survival by Delivering BDNF

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Introduction: The passage of adeno-associated virus (AAV) across the integrated blood brain barrier (BBB) is difficult in adults, which limits the application of AAV gene therapy vectors in central nervous system (CNS) diseases. Thus, developing a method to increase AAV entry and transduction in CNS is necessary. Hypothermia is a condition in which an organism's temperature drops below that required for normal metabolism and functions. In accordance with central body temperature, hypothermia is divided into four levels, including mild (32-35°C), moderate (28-32°C), severe (20-28°C) and profound (below 20°C). Hypothermia has been applied in clinic to treat spinal cord injuries (SCI) as an aid for neuroprotection but increasing permeability of BBB is considered as its side effect. Therefore, we utilized hypothermia to induce temporary BBB permeability and improve AAV passage into CNS. Moreover, we applied the short-term hypothermia to increase AAV delivery with brain-derived neurotrophic factor (BDNF) into CNS for the treatment of amyotrophic lateral sclerosis (ALS) in an animal model. **Results:** We found that short-term exposure to moderate or severe hypothermia enhanced AAV transduction into gray matter of spinal cord by 11.3-12.8 folds in adult mouse (Fig. 1A). Moreover, the distribution of gene expression was quite uniform along the length of the spinal cord throughout from the cervical, thoracic to lumbar segments. We observed that hypothermia increased AAV infection in neurons primarily, but not glial cells. Furthermore, application of the short-term moderate hypothermia increased greatly AAV9-mediated brain-derived neurotrophic factor

(BDNF) gene expression in spinal cord, which significantly delayed the disease onset and increased the survival of ALS mice (Fig. 1B). In contrast, AAV9 systemic delivery without hypothermia did neither postpone ALS onset nor impact survival, although skeletal muscle function improved partially. **Conclusion:** Short-term moderate or severe hypothermia could transiently increase BBB permeability, and promote AAV vector passage and enhance the target gene expression in adult CNS, especially in neurons. Overall, the combination of short-term moderate hypothermia with AAV systemic injection provides a novel method for inducing high expression of therapeutic genes for CNS diseases.

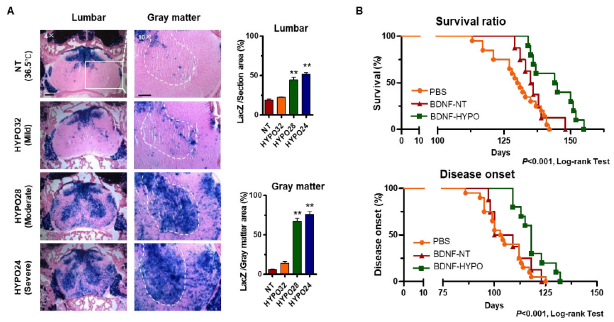

Fig. 1 A. Effect of different temperature gradient of hypothermia on AAV9-mediated transduction into spinal cord. **P<0.01 vs. NT group. B. Effect of AAV-mediated BDNF expression with or without hypothermia on the survival and muscle function of ALS mice.

962. Targeting Muscle Satellite Cells with Adeno-Associated Viral Vectors

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Adeno-associated viral (AAV) vectors are currently amongst the most frequently used delivery tools for gene therapy. AAV delivery of therapeutic transgenes or gene editing tools for treatment of muscle diseases, such as muscular dystrophies, have yielded encouraging results in various animal disease models. Although AAV efficiently targets muscle fibers, the extent of transduction in the muscle stem cell population, termed satellite cells, is unclear. Satellite cells reside in a specialized niche between the basal lamina and sarcolemma of individual muscle fibers and account for ~5% of all muscle nuclei. Because satellite cells continuously replenish skeletal muscle in response to tissue damage, the genetic correction of a population of these self-renewing cells could generate a sustained source of therapeutic gene production. In fact, because episomal AAV vectors are lost by dilution following cell division, permanent correction of the genomic copy of mutated genes in proliferating satellite cells is a particularly compelling advantage of gene editing technologies. Furthermore, efficient targeting of satellite cells with AAV vectors *in vivo* would enable many studies of the function and regulation of satellite cell biology within the native environment. To investigate AAV transduction of satellite cells, we used Pax7-nGFP mice, in which nuclear localized GFP is knocked into the first exon of Pax7 that is specifically expressed in satellite cells. Using AAV-based delivery of CRISPR/Cas9 gene editing tools targeted to the dystrophin locus in a mouse model of Duchenne muscular dystrophy, we detected gene editing in satellite cells, reproducing recently published results (Tabebordbar, *Science* 2016). These studies were performed with AAV8; however, several other AAV serotypes are under preclinical development for delivery to skeletal muscle. Moreover, commonly used serotypes in the gene therapy community have not been screened for tropism to muscle satellite cells. Therefore, we assessed satellite cell transduction efficiency with a panel of AAV serotypes using a dual reporter mouse to permanently mark satellite cells transduced by AAV encoding Cre recombinase. We crossed Pax7-nGFP mice with Ai9 mice, which express CAG-loxP-STOP-loxP-tdTomato at the Rosa26 locus. Cre-mediated recombination removes the stop cassette to allow for tdTomato expression in cells transduced by AAV. Therefore, any GFP+ cells that are also tdTomato+ mark satellite cells that were transduced with the AAV vector. We injected AAV1, AAV2, AAV5, AAV6.2, AAV8, and AAV9 serotypes encoding Cre into dual reporter mice at equivalent doses of 4.72E+11 viral genome copies for intramuscular injections or 2E+12 viral genomes for systemic injections. Following intramuscular injection into the tibialis anterior muscle, AAV9 and AAV8 marked the Pax7-GFP+ cells most efficiently, at 60% and 54%, respectively, as determined by flow cytometry analysis (n=5 muscles, mean + SEM). Systemic injections of AAV9 also displayed significant marking of Pax7-GFP+ cells, ranging from 19-31% for tibialis anterior, soleus, quadriceps, diaphragm, and triceps (n=5 mice, mean + SEM). Ongoing experiments include assessing Pax7+ cell targeting by novel engineered AAV serotypes and the contribution of these AAV-transduced cells to muscle regeneration through self-renewal. This work will determine the AAV serotype with maximal tropism for satellite cells in order to inform optimal AAV delivery strategies to provide long-term therapeutic gene expression in skeletal muscle while maintaining the safety advantages of using a non-integrating viral vector.

963. Development of a Novel Recombinant Adeno-Associated Virus Production System Using Human Bocavirus 1 Helper Genes

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Previously, we reported that human bocavirus 1 (HBoV1), a novel autonomous parvovirus, is a helper virus supporting wild-type adeno-associated virus (WT AAV) replication in well-differentiated/polarized primary human airway epithelium. We also demonstrated that HBoV1 genes *NP1*, *NS2*, and *BocaSR* (the bocavirus small noncoding RNA) are the minimal components for WT AAV replication in 293 cells and HeLa Cells. In this study, we compare the helper functions from HBoV1 with that from adenovirus for the production of recombinant AAV vector (rAAV) in 293 cells. We demonstrate that triple plasmids transfection of 1) a cloned HBoV1 minigenome (pBocaHelper) that expresses HBoV1 genes *NP1*, *NS2*, and *BocaSR*, 2) pAAV transfer plasmid, and 3) pAAVRepCap supports rAAV production in 293 cells. Despite a production yield of 1-2 log lower than that using pAdHelper (expressing adenovirus genes *E2A, E4 and VAI*), rAAV vector produced by pBocaHelper transduced cells as efficiently as that produced by pAdHelper. To optimize this novel rAAV production system, we examined the expression of AAV proteins and the replication of rAAV genome supporting by the transfection of pBocaHelper. We found that the low vector production is largely due to inefficient expression of the AAV Rep52 and capsid proteins, as well as reduced rAAV

genome replication. When the AAV P19 promoter in pAAVRep was replaced by or the AAV capsid proteins were expressed under the stronger CMV promoter, the enhanced expression of Rep52 and capsid proteins significantly improved the rAAV production by pBocaHelper, approaching a level of 50-70% of that produced by pAdHelper. Through further dissection of the helper functions from pAdHelper, we found that an addition of the Ad *E2A* gene with pBocaHelper significantly increased rAAV DNA replication. As a result, the rAAV vector production reached a level of two times higher than that using pAdHelper in 293 cells in the context of overexpression of capsid proteins. Finally, based on the above findings, we combined HBoV1 *NP1* and *NS2* genes with pAdHelper to create a novel helper plasmid named as pSuperHelper, for which overexpression AAV genes *rep* and *cap* by the CMV promoter is not necessary. rAAV production from the new system using the pSuperHelper is ~2-fold greater than that from the conventional production system using pAdHelper. This system may also enable production of rAAV in alternative non-293 cell line, further expanding the capabilities of large-scale rAAV production.

Advancements in T Cell-Based Therapies

964. Abstract Withdrawn

965. Effective Antitumor Responses in the Absence of Toxicity in Pancreatic Ductal Adenocarcinoma Models by Targeting B7-H3 via Chimeric Antigen Receptor T Cells

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The success of chimeric antigen receptor T cells (CAR-Ts) in solid tumors remains challenging because tumor-associated antigens targetable by CAR-Ts are limited. B7-H3 is a type I transmembrane protein aberrantly expressed in 63% to 94% of tumor cells in different cancers, while of limited expression in normal tissues. We elected pancreatic ductal adenocarcinoma (PDAC) as a tumor model to investigate the efficacy and safety of targeting B7-H3 with CAR-Ts. We generated B7-H3.CARs, using the scFv derived from the anti-human B7-H3 mAb 376.96, and encoding either CD28 (B7-H3.CAR-28ζ-Ts) or 4-1BB (B7-H3.CAR-BBζ-Ts) co-stimulatory endodomains. Transduction efficiency of CAR-Ts was >60% (n=22). Antitumor activity of control cells (Non-transduced, NTs) or B7-H3.CAR-Ts was assessed against B7-H3$^+$ PDAC cell lines (Panc-1, BxPC-3, Panc-10.05, Capan-1, and AsPC-1) in co-culture assays at a T cell:tumor cell ratio of 1:5. After 5 days co-culture, tumor cells were completely eliminated by B7-H3.CAR-Ts but not NTs ($p<0.0001$). Cytolytic activity of B7-H3.CAR-Ts was corroborated by IFNγ (>2000pg/mL/10^5 cells) and IL-2 (>800 pg/mL/10^5 cells) release. We next implanted orthotopically into the pancreas of NSG mice Firefly luciferase$^+$ Panc-1 cells (2×10^5 cells/mouse). Two weeks later, when the median tumor bioluminescence intensity (BLI) was $1.5\pm1.5\times10^9$, mice received CD19.CAR-Ts (Ctrl-Ts), B7-H3.CAR-28ζ-Ts or B7-H3.CAR-BBζ-Ts (1×10^7 cells/mouse, n=5/group). Both B7-H3.CAR-28ζ-Ts and B7-H3.CAR-BBζ-Ts effectively controlled tumor growth and all mice remained tumor free up to 80 days post-treatment. Antitumor activity of B7-H3.CAR-Ts was confirmed also in a high tumor burden model, by delaying CAR-Ts infusion until day 28 post Panc-1 tumor cell implantation, when the median BLI of tumors was $2.7\pm1.3\times10^{10}$. Also in this case, B7-H3.CAR-Ts effectively controlled the tumor growth, with 4/5 B7-H3.CAR-28ζ-Ts treated mice and 3/5 B7-H3.CAR-BBζ-Ts treated mice tumor free 68 days after treatment (n=5/group). Antitumor activity of B7-H3.CAR-Ts was confirmed in PDAC-patient derived xenograft (PDX) orthotopic NSG mice, where 6/10 mice treated with B7-H3.CAR-Ts were tumor free 85 days after treatment. Finally, since the B7-H3.CAR we have generated cross-reacts with murine B7-H3, we studied the efficacy and safety of B7-H3.CAR-Ts in an immunocompetent mouse model in which C57BL/6J mice were orthotopically engrafted with the mB7-H3$^+$ murine PDAC tumor cell line KPC-4662. After 17 days, mice were irradiated with 400 cGy and then infused with syngeneic Ctrl-Ts or mB7-H3.CAR-Ts. mB7-H3.CAR-Ts controlled tumor growth for >4 weeks, as assessed by tumor weight (286±103 mg vs 140±27 mg, n=5, $p=0.016$) on day 33-34 post-treatment when mice were euthanized. Two of the five control mice also showed liver metastases, while no metastases occurred in mB7-H3.CAR-Ts treated mice. A comprehensive analysis of mice treated with mB7-H3.CAR-Ts showed normal counts of hematopoietic cells in blood, spleen and bone marrow. No tissue damage in any analyzed organs was observed, further supporting the safety and efficacy of CAR-Ts targeting the B7-H3 molecule for clinical application.

966. Car T Cells Secreting an Immune Checkpoint Blockade scFv Have Enhanced Anti-Tumor Efficacy

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Chimeric antigen receptor (CAR) T cells are genetically engineered to recognize tumor cells and CAR T cell therapy has had impressive results in the setting of B cell malignancies but has been less effective in treating solid tumors. This may be in part due to the inhibitory tumor microenvironment (TME) of solid tumors, including expression of ligands that bind inhibitory receptors on T cells, e.g. programmed death receptor 1 (PD-1), that can dampen CAR T cell responses. To prevent PD-1-mediated dampening of CAR T cell function, we have utilized our armored CAR platform to co-modify CAR T cells. Utilizing PD-1 blockade as a proof-of-principal, we hypothesized that armored CAR T cells that secrete a scFv blocking the interaction between PD-1 on immune cells and PDL1 on tumor cells will work in an autocrine manner to prevent suppression of CAR T cells and in a paracrine manner to un-inhibit bystander tumor-specific immune cells. Given our hypothesized mechanisms of action, the ideal method to test

this technology is in an immunocompetent syngeneic mouse model. CAR constructs were engineered directed against MUC-16 (ecto) and an anti-mouse PD-1 scFv. Mouse T cells transduced with these constructs expressed the CAR on the surface and secreted detectable amounts of scFv that bound to mouse PD-1. The scFv-secreting CAR T cells were cytotoxic and produced IFN-γ when co-cultured with PD-L1 expressing tumors *in vitro*. We utilized a syngeneic, immune-competent mouse model of metastatic ovarian carcinoma to study scFv secreting CAR T cells in a model with an intact TME. In tumor-bearing mice treated with CAR T cells, scFv-secreting CAR T cells enhanced survival as compared to second generation CAR T cells. The survival benefit achieved with scFv-secreting CAR T cells was comparable to that achieved with systemic infusion of PD-1 blocking antibody, but with localized delivery of PD-1 blockade. Mice treated with scFv-secreting CAR T cells had detectable scFv *in vivo* in the TME. Lastly, long term surviving mice had detectable CAR T cells and had enhanced survival when rechallenged with tumor, demonstrating CAR T cell persistence and an immunological memory. We next aimed to translate PD-1 blocking scFv CAR T cells to a clinically relevant human model utilizing a novel anti-human PD-1 blocking scFv. Human T cells modified with the CAR constructs express the CAR on the surface and secrete detectable amounts of PD-1 blocking scFv. The scFv binds to human PD-1 and scFv-secreting CAR T cells are cytotoxic to PD-L1 expressing tumors. Expression of PD-1-blocking scFv enhances CAR T cell function against PD-L1 expressing tumors in xenograft models of solid tumors by enhancing survival in tumor-bearing mice as compared to second generation CAR T cells. Furthermore, scFv-secreting CAR T cells exhibit *in vivo* bystander T cell enhancement of function, suggesting scFv-secreting CAR T cells can reactivate endogenous TILs in the TME. Ongoing experiments are aimed at elucidating the pharmacokinetics of scFv secreted by CAR T cells as compared to checkpoint blockade antibody given alongside CAR T cells. These data support the novel concept that localized delivery of scFv by CAR T cells can successfully block PD-1 binding to PD-L1 to enhance the overall anti-tumor efficacy of CAR T cell therapy.

967. Early Signs of Clinical Activity in Aml Patients Receiving NKG2D CAR T Cell Therapy in the Absence of Pre-Conditioning Chemotherapy: An Alternative Strategy to CAR T Cell Therapy

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NKG2D is a receptor with specificity for a broad range of stress ligands (MICA, MICB and ULBP1-6) that are expressed on a variety of tumors. Extensive syngeneic mouse studies carried out by Professor Charles Sentman (Dartmouth College) concluded that T cells bearing a chimeric antigen receptor (CAR) consisting of the fusion of the NKG2D receptor with the CD3ζ signaling domain could drive profound anti-tumor activity in a broad range of tumor models. Importantly, this CAR T cell approach involved multiple infusions of the T cells in the absence of pre-conditioning chemotherapy, a paradigm that differs from many current CAR T cell therapies. Our recent pre-clinical studies have shown that three infusions of human CYAD-01 T cells can control the growth of the highly aggressive THP-1 AML cell line in the NSG mouse while a single infusion has a more transient effect. In agreement with the previous syngeneic mouse studies, the in vivo persistence of CYAD-01 CAR T cells remains short. Based upon this work, phase I clinical studies testing this NKG2D CAR T cell (called CYAD-01) approach have been initiated. In our ongoing clinical trial THINK (NCT03018405), three infusions of CYAD-01 at weekly intervals are given to patients with no pre-conditioning or other supportive therapy. Encouragingly, at the first dose level (i.e. $0.3*10^9$ cells/injection), one relapsed AML patient reached a morphologic leukemia-free state (MLFS) after the CYAD-01 treatment, and resolution of symptoms with improved hematopoiesis at 3 months follow up at which time he underwent an allo-graft. At 3 months post-transplant, the patient is in complete remission with 100% donor chimerism. The dose escalation continues in both hematological cancer but also in parallel studies in colorectal cancer. As seen in similar trials, there is a large variation in T cell phenotype between patients. However, in terms of post-infusion analysis, we did observe a relative increase in the systemic levels of SDF-1 and RANTES in the patient who achieved MLFS but no evidence of modulation of other systemic cytokines or chemokines. These results demonstrate the on-going translation of CYAD-01 with interesting early signs of activity in patients with advanced refractory cancer. Continuing to understand the mode of action of CYAD-01 CAR T cells remains essential as well as considering whether combinations of therapy may potentially synergize with CYAD-01.

968. Highly Efficient and Specific Multiplexed Gene Editing in T Cells Using Enhanced Zinc-Finger Nucleases (Zfns) Enables Strategic Engineering of Allogeneic T Cell Immunotherapies

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Despite promising clinical data and the great therapeutic potential of T cells expressing CD19-directed chimeric antigen receptors (CARs) in the treatment of hematological malignancies, immunotherapy with autologous CAR-T cells faces some challenges, particularly in product consistency, scalability and affordability. Alternatively, engineered allogeneic CAR-T cells generated from healthy donors, lacking endogenous TCR and HLA class-I expression, would enable off-the-shelf CAR-T therapy of a well-characterized consistent product that could be administered to a broad population of patients on demand. To pursue this approach, highly efficient and precise gene editing capability is imperative to generate the desired T cell product profile:

TCR/CD3-negative achieved by knocking out T-cell receptor alpha constant region (TRAC), HLA class I-negative achieved by disrupting HLA complex formation by knocking out the β2-microglobulin (β2M) gene, and positive expression of a gene of interest (TCR/ CAR) achieved by site-directed gene insertion into the disrupted TRAC locus. Using ZFNs, we have developed multiplexed gene editing capabilities that can simultaneously disrupt the TRAC and β2M loci at a double knock-out (KO) efficiency of greater than 90%, and achieve targeted insertion of GFP into TRAC at greater than 90%. Furthermore, a rigorous and deep molecular interrogation of the ZFN-treated T cells revealed undetectable off-target nuclease activity, highlighting the specificity of our enhanced ZFN technology. Efficiency is critical in multiplexing to generate a higher proportion of final cells with all the desired edits. For example, a product that has 2 genes disrupted at 90% and 1 gene inserted at 90%, the compound efficiency of cells with all 3 successful edits would be 73%. However, if the efficiency of all three gene edits dropped to 70%, a mere 34% of the final cell product would contain all 3 edits. We have reported up to 99.5% TCR KO in healthy donor T cells and up to 96% β2M KO. In addition, we have tested ZFN reagents to disrupt checkpoint genes, such as CISH, at greater than 90% efficiency. Checkpoint gene KO may enhance the persistence and efficacy of allogeneic CAR-T cells in specific indications based on emerging biomarker analyses from ongoing CD19 CAR-T trials. Thus, additional multiplexing to knock-out CISH or PD-1, in addition to TRAC, β2M and a gene insertion (TCR/CAR), would enable more strategic allogeneic T cell product development. We have recently achieved a compound efficiency of 76% in T cells, edited in a single step to knock-out three genes by ZFNs, TRAC, β2M and CISH, at efficiencies of 93%, 96%, 93%, respectively, with simultaneous AAV treatment for GFP insertion into TRAC at 91% efficiency. In summary, we are reporting highly efficient and reproducible multiplexed gene editing capability, enabling the generation of a healthy product in which ~76% of T cells contain all 4 desired edits (3 gene KO and 1 targeted gene insertion). These industry leading capabilities enable the development of strategic, defined and well-characterized allogeneic CAR-T cell products for application in oncology.

969. Armored Glypican-3-Specific CAR T Cells for the Immunotherapy of Hepatocellular Carcinoma

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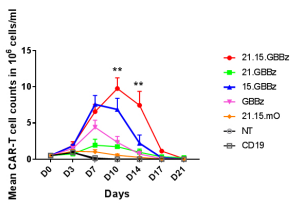

Figure 1. Expansion and survival of GPC3-CAR T cells *in vitro*. GPC3-CAR T cells were co-cultured with GPC3-positive Huh-7 HCC cell line every 3-4 days. Absolute number of CAR T cells is shown at indicated time points. ** *P* value < 0.01, ANOVA.

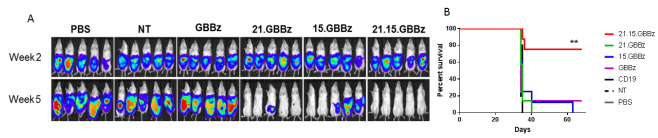

Figure 2. A. GPC3-CAR T cells co-expressing IL-21 and/or IL-15 (21.GBBz, 15.GBBz and 21.15.GBBz) induced more robust anti-tumor activity versus GPC3-CAR T cells co-expressing no cytokines (GBBz). NSG mice were injected 2x10^6 Huh-7.Ffluc tumor cells IP followed by the IV injection of 2x10^6 GPC3-CAR T cells or non-transduced T cell as control after two weeks. **B. GPC3-CAR T cells co-expressing both IL-15 and IL-21 (21.15.GBBz) have superior antitumor activity resulting in a survival advantage over GPC3-CAR T cells co-expressing single/no cytokines (21.GBBz, 15.GBBz and GBBz).** NSG mice were injected 2x10^6 Huh-7.Ffluc tumor cells IP followed by the IV injection of 0.5x10^6 GPC3-CAR T cells or non-transduced T cell as control after one week. ** *P* value = 0.0012, Logrank test.

Hepatocellular carcinoma (HCC) is the sixth commonest cancer accounting for over half million annual diagnoses and the third most common cause of cancer related mortality worldwide. Approximately 20,000 new HCC cases are reported in United States every year and more than 60% patients die from the disease. Thus, novel approaches are needed to improve outcomes in patients with HCC and immunotherapy may fill this need. Glypican-3 (GPC3) is an attractive immunotherapeutic target as it is not expressed on normal adult tissue but is highly expressed in HCC (>70%). We systematically tested GPC3 specific Chimeric Antigen Receptors (CARs; GPC3-CAR) harboring endodomains derived from CD28, 4-1BB or both and found that CAR T cells expressing a 4-1BB endodomain (GBBz) exhibited the most promising antitumor activity. Treatment of solid tumors using traditional CAR T cells however has yielded only modest clinical benefit, therefore, additional strategies are needed to improve CAR T cells' antitumor activity. The absence of homeostatic cytokines such as interleukin 15 (IL-15) in solid tumors is associated with defective lymphocyte activation and decreased patient survival. Thus, we hypothesized that transgenic expression of IL-15 in GPC3-CAR T cells will enhance their antitumor effect. Moreover, since IL-15 and IL-21 can act synergistically to further promote T cell expansion and function, we also hypothesized that co-expression of both IL-15 and IL-21 by GPC3-CAR T cells will further improve their antitumor effect. Armored GPC3-CAR T cells co-expressing IL-15 and/or IL-21 (15.GBBz, 21.GBBz and 21.15.GBBz) efficiently killed GPC3-positive tumor cells and produced effector cytokines GM-CSF, IFNγ, TNFα, IL-2, IL-10, IL-17, IL-13 and IL-4 *in vitro*. Co-expression of both IL-15 and IL-21 improved production of Th1 effector cytokines, while inhibiting the Th2 cytokine IL-13 (p <0.001, ANOVA). IL-15 co-expression preferentially expanded CD8+ GPC3-CAR T cells, following activation (p <0.001, ANOVA). To examine the proliferation potenatial of GPC3-CAR T cells which is associated with increased therapeutic efficacy, T cells were co-cultured with fresh tumor cells repeatedly every 3-4 days. We found that IL-21 and IL-15 co-expression enhanced GPC3-CAR T cell expansion and persistence compared to GBBz alone, 15.GBBz or 21.GBBz (Fig. 1). We also examined the antitumor efficacy of IL-15 and/or IL-21 armored GPC3-CAR T cells in HCC xenograft murine models. IL-21 and IL-15 co-expressing GPC3-CAR T cells had robust antitumor activity superior to all the other constructs tested (Fig. 2; p=0.0012, Logrank test). In summary, these studies indicate that co-expression of both IL-15 and IL-21 improves antitumor properties of GPC3-CAR T cells and provides rationale for clinical testing of this strategy in patients with HCC.

970. Vector Integration and Efficacy of CD19-Directed CAR T Cell Therapy in ALL and CLL

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Chimeric antigen receptor-engineered T-cells (CAR T cells) provide a breakthrough for personalized cancer therapy. In this method, a gene encoding a chimeric antigen receptor is delivered into patient T-cells *ex vivo* using a lentiviral vector, after which cells are reinfused into patients. In CART 19 therapy, the engineered T-cells attack and destroy CD19 positive cancer cells. While dramatically successful for some treatment-refractory cancers, a significant proportion of patients do not experience therapeutic levels of CAR T cell expansion--thus it is important to investigate factors driving successful expansion in responders in more detail. Here we have analyzed sites of lentiviral vector integration in CAR T cells from trials to treat ALL and CLL, comparing successful and unsuccessful therapy in longitudinal data sets for 40 subjects. The location of each integrated vector marks a cell lineage uniquely, allowing monitoring of the population biology of gene-modified T-cells. We found that relatively larger and more diverse populations of modified T-cells were associated with improved outcome. Vector integration can also modify activity of nearby genes, as we recently reported for an integration event in the DNA methylcytosine dioxygenase gene TET2, where clonal expansion was associated with successful therapy. Insertional mutagenesis was evaluated here over five criteria, including i) clonal expansion after infusion, ii) increasing frequency of unique integration sites per gene after infusion, iii) development of orientation bias, iv) longitudinal persistence, and v) accumulation of integration site clusters. Analysis disclosed genes and cell pathways associated with superior cell proliferation and persistence. These data thus provide multiple approaches for improvement of manufacturing CAR T cells.

971. Cancer Immunotherapy with APOBEC3B-Induced Heteroclitic Library Tumor Cell Vaccines and Immune Checkpoint Blockade

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We have previously shown that a Vesicular Stomatitis Virus-based cDNA library expressing xenogeneic altered self-epitopes led to T cell based rejection of both prostate cancer, glioma and melanoma. The use of a library of altered self-peptides which reflects the transcriptome of the tumor circumvents the necessity for *a priori* knowledge of which antigens may be immunogenic in a patient. We have expanded this concept of altered-self library vaccination using whole tumor cell vaccines modified by the overexpression of APOBEC3B, a cytosine deaminase which generates C to T transitions, and to a lesser frequency C to G transversion mutations. We hypothesized that the ability of APOBEC enzymes to generate mutations in an unbiased approach throughout the genome would generate an altered-self library vaccine. Consistent with this, a freeze-thawed whole tumor cell vaccine prepared from B16 melanoma cells stably overexpressing human APOBEC3B treated established subcutaneous parental B16 tumors and, when combined with PD-1 checkpoint blockade, cured between 75%-100% of mice. Depletion of either CD4 or CD8 cells abrogated therapy. Moreover, T cells from mice treated with the vaccine and anti-PD1 produced high levels of interferon (IFN)-γ when co-cultured with parental unmodified B16 melanoma cells, correlating with therapeutic activity. Whole genome sequencing of B16 APOBEC3B overexpressing cell lines identified 301 C to T or G to A missense mutations unique to the APOBEC3B line. Using an *in silico* MHC binding affinity algorithm for peptides whose binding affinity for $H2K^b$ or $H2K^d$ was below a threshold of 500 nM, 10 high affinity APOBEC3B- induced heteroclitic peptides were identified and the ability of the B16 cells modified by APOBEC3B to prime T cells against these heteroclitic peptide candidates was experimentally validated *in vitro*. We have also shown that this approach can be extended to an orthotopic brainstem model of High Grade Glioma. Thus, GL261 cells stereotactically implanted into the brainstem were significantly responsive to treatment with an APOBEC3B- modified GL261 vaccine in combination with anti-PD-1 checkpoint blockade and the priming of a T cell response to the APOBEC3B- modified GL261 vaccine was confirmed *in vitro.* In summary, our data show that, when overexpressed in tumor cells, APOBEC3B generates a library of heteroclite sequences which primes both CD4 and CD8 T cells that have escaped central tolerance and which recognize both newly mutated antigens from the vaccine, as well as the corresponding unaltered self epitopes expressed on the tumor cells. This approach represents a potent new method to develop neo-epitope based vaccine and we are currently developing such a strategy for the treatment of pediatric High Grade Glioma.

Adenovirus Vectors and Other DNA Virus Vectors, Technical Advances in CNS Gene Therapy

972. HDAd5/35++-Mediated Targeted Integration in HSCs of AAVS1 Transgenic Mice Results in Efficient Transgene Marking in Peripheral Blood Mononuclear Cells

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A major task in the HSC gene therapy field is targeting transgene integration to pre-selected sites, so-called "Safe Harbor Loci" through homology-dependent repair (HDR) mechanisms. The adeno-associated virus integration site 1 (AAVS1) locus is the best characterized of these loci. We have evaluated helper-dependent HDAd5/35++ adenovirus vectors for targeted integration in AAVS1 transgenic mice. HDAd5/35++ vectors target CD46, a receptor that is expressed on primitive HSCs. The first HDAd5/35++ vector expressed an AAVS1-specific CRISPR/Cas9 (HDAd-CRISPR). The HDAd5/35++-based donor vector contained a GFP/mgmt transgene cassette flanked by AAVS1 homology regions and AAVS1-CRISPR cleavage sites (HDAd-donor). Expression of AAVS1 CRISPR will create double-strand DNA breaks in the chromosomal AAVS1 site, and also release the donor cassette from the HDAd-donor vector to increase the efficacy of HDR. Target site-specific CRISPR/Cas9 cleavage and efficient donor cassette release was demonstrated after HDAd-CRISPR + HDAd-donor co-transduction of bone marrow lineage-negative (lin^-) cells from AAVS1/hCD46-transgenic mice. Co-transduced lin^- cells were then transplanted into lethally irradiated C57Bl/6 recipients. Engraftment rates were nearly 100% indicating that HDAd5/35++ vector transduction did not affect mouse HSCs. Four weeks after transplantation, three cycles (with an interval of 2 weeks) of *in vivo* selection for transduced HSCs with low dose O^6BG/BCNU were started. At week 12 after transplantation, 30-65% of PBMCs expressed GFP in individual mice. These marking rates were confirmed in bone marrow HSCs. In progenitor colony assays with bone marrow lin^- cells on average 40% of colonies were GFP-positive. In-out PCR on colonies showed site-specific integration in the majority of colonies. GFP marking in PBMCs of secondary transplant recipients was on average 50%. No GFP-positive PBMCs were found in primary or secondary animals that were transplanted with lin^- cells transduced with HDAd-donor or HDAd-CRISPR alone. The HDAd5/35++ vector system is a new tool for achieving targeted transgene integration in HSCs. Notably, HDAd5/35++ vectors have an insert capacity of 30kb which allows incorporating large stretches of homology. This, in turn, should increase the efficacy of HDR and targeted integration. We are currently testing this hypothesis.

973. Molecular Evolution of the Next Generation of Bocaviral Vectors

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Human Bocavirus 1 (HBoV1) is a parvovirus that was originally identified in human airways over a decade ago. In 2013, it was reported that HBoV1 can encapsidate single-stranded AAV genomes, yielding HBoV1/AAV2 chimeras that specifically transduce polarized human airway epithelia (pHAE) culture *in vitro*. Most recently, we expanded the repertoire of HBoV/AAV chimeras by assembling the capsid genes of four additional primate human bocaviruses, HBoV2-4 and Gorilla Bocavirus (GBoV). Here, we present new data from their functional analyses in pHAE and human lung organoids showing that not only HBoV1, but also the other bocaviral variants transduce these cells, albeit with varying efficiencies (HBoV1/GboV>HBoV4>HBoV2/3). Interestingly, flow cytometry and immunostaining of HAE showed that HBoV1 and GBoV mainly target ciliated cells and to a lesser extent club cells within human airways, illustrating the unique cell specificity of bocaviruses and their great potential for precise *in vivo* gene delivery. In the past, we and many others employed molecular evolution of AAV capsids via DNA family shuffling, to create and select synthetic capsids with novel properties. Here, for the first time, we adapted this powerful technology to primate bocaviruses, with the aim to breed the next generation of bocaviral vectors with increased efficiency and customized specificity. To this end, we shuffled the five parental serotypes (HBoV1-4, GBoV), yielding large and highly diverse capsid libraries (>$5x10^7$ variants each) one of which we then cycled multiple times in pHAE for proof-of-concept. After each round, enriched *capsid* genes were sequenced to track molecular evolution and concurrently subcloned for production of a new library. Remarkably, already after one selection round, we observed a shift in the library composition towards a site-specific accumulation of a stretch derived from HBoV4. Two further rounds led to an additional enrichment of HBoV1 or HBoV2 variable regions at the C terminus, as well as a portion of HBoV3 at the very end of the capsid gene. In contrast, the N terminus of the capsid was least affected by the selection pressure, in line with the high degree of conservation of the embedded phospholipase domain. Notably, we observed a nearly 300-fold increase in library viral titers after the first selection round (3e9 vs. 9e11 viral genomes/ml), most likely reflecting the expected substantial accumulation of assembly-competent virions during molecular evolution. Here, by building on our profound experience with AAV vectors, we have successfully applied DNA family shuffling to expand the repertoire of bocaviral vectors. We

believe that our new libraries will greatly assist in dissecting the biology of Bocavirus infection and in selecting novel vectors for specific gene delivery to the airways and beyond.

974. A Human In Vitro Model to Study Adenoviral Receptors and Virus-Cell Interactions

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Adenoviral receptors are studied especially since adenoviruses have been scoped as potential vectors for gene therapy. To develop adenoviral cell- or tissue-specific gene delivery, understanding of the attachment and infection mechanisms of adenoviruses is crucial. Several adenoviral attachment proteins such as CD46, CAR and sialic acid have been identified and studied. However, recent studies had two major limitations. First, they have mostly been performed in non-human cells and second, no systematic analysis of receptor usage comparing different adenoviruses side-by-side has been performed. By combining our GFP/luciferase tagged adenovirus library including all adenovirus species (Zhang et al., Cell Reports 2017) with a human gene knockout model, we can overcome limitations and generate data on adenoviral receptor usage in a systematic approach. We used the CRISPR/Cas9 system for transfer of the customized CRISPR/Cas9 machinery to perform a knockout of the CD46 and CAR cell surface receptors in the human lung epithelial carcinoma cell line A549. Functionality of the CRISPR/Cas9 system was verified by T7E1 assay which revealed specific disruption of the target gene. Knockout cells were sorted by magnetic cell sorting or by co-transfection with a linear donor DNA encoding a neomycin resistance cassette and subsequent selection with neomycin. Single cell clones were amplified and CD46 and CAR knockouts were measured by flow cytometry analysis which showed complete loss of CD46 and CAR on the cell surface confirming single- and double-knockouts of these receptors on a human cell line. Next human CD46 knockout cells were infected with luciferase tagged adenoviruses AdV5, AdV10, AdV35 and AdV50. Virus uptake was measured with luciferase assay and compared with wildtype cells. We found that the CD46 surface protein is essential for virus uptake of AdV35 and AdV50 but not of AdV5 and AdV10. We are currently screening our complete tagged adenovirus library on single and double-knockout cell lines. In summary, we established three stable human knockout cell lines with disruption of CD46 and CAR only and one cell line carrying a double knockout of CD46 and CAR. Further studies will involve further depletion of sialic acid as a third cellular adenovirus attachment molecule. We believe that our human knockout cell model will help to identify most applicable adenovirus types for gene therapy and to further understand the infection biology of adenoviruses.

975. Novel Small Molecules That Enhance Adenovirus Transduction of the Airway Epithelium

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Adenovirus remains one of the most common vector system used in gene therapy clinical trials worldwide and several adenoviral vectors show promise in phase III clinical trials. One major limitation to the efficacy of adenovirus-mediated gene therapy is low transduction due to the limited primary receptor availability in most tissues. Interventions able to boost adenovirus transduction would have significant implications for both improving transduction and lowering the viral dose below the immunogenic threshold. The primary receptor for most adenovirus serotypes is the Coxsackievirus and adenovirus receptor (CAR). While the CAREx7 isoform localizes on the basolateral side of epithelial cells, the CAREx8 isoform is present on the apical side where it can facilitate adenovirus entry into the airway epithelium. MAGI-1, a cellular scaffolding protein, has been shown to regulate CAREx8 expression via two of its PSD-95/Dlg/ZO-1 (PDZ) domains, PDZ1 and PDZ3. It has been demonstrated that the PDZ3 domain of MAGI-1 promotes the degradation of CAREx8, while MAGI-1 PDZ1 protects CAREx8 from degradation. We have found that blocking the interaction between MAGI-1 PDZ3 and CAREx8 with small cell-permeable peptides can increase CAREx8 expression and thus adenovirus transduction. However, the peptides are based on known MAGI-1 PDZ3 interactors, such as CAREx8, and are expected to have the same affinity as endogenous interactions. We hypothesize that we can reduce binding competition and the dose of peptide required with higher affinity peptides. We utilized peptide design algorithms to create new peptides that bind MAGI-1 PDZ3 with a significantly higher affinity. Using the PinaColada algorithm, we chose an input structure of MAGI-1 PDZ3 bound to the C-terminus of CAREx8 and systematically mutated the residues of CAREx8 to determine peptides that bind with a higher affinity. All candidate protein-peptide interactions designed were validated using in silico docking simulations. We will test these peptides in vitro and in vivo for their efficacy in increasing AdV transduction. These small peptide inhibitors have the ability to transiently increase adenovirus-mediated gene transfer and offer the potential to increase the efficacy of adenovirus-mediated gene therapy.

976. Allele-Specific RNA Interference: Precision Gene Therapy for Dominant Forms of Charcot-Marie-Tooth Disease

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Charcot-Marie-Tooth Disease (CMT) is a heterogeneous group of inherited peripheral neuropathies affecting 1:2500 individuals worldwide. To date, fifteen different mutations in *GARS (*glycyl-tRNA synthetase*)* have been identified in patients with autosomal dominant CMT Type 2D (CMT2D). Although the mechanisms through which mutant forms of GARS cause axon degeneration remain controversial,

preliminary data from CMT2D patients and mouse models of the disease (*Gars*$^{C201R/+}$ and *Gars*$^{P278KY/+}$) suggest that the expression of mutant GARS may cause toxic gain-of-function effects in peripheral nerves. As such, the selective silencing of mutant *GARS* expression should benefit patients with this disorder. In response, we have developed a gene therapy strategy that reduces the expression of mutant *Gars* transcripts through allele-specific RNAi. Specifically, self-complementary adeno-associated viral vectors (scAAV9) expressing therapeutic microRNAs were engineered to specifically target mutant *Gars* transcripts for degradation. Preliminary data show that when delivered after disease onset, our gene therapy can significantly alleviate symptoms of neuropathy in an established mouse model of CMT2D (*Gars*$^{P278KY/+}$) confirming the proof-of-concept of our therapeutic strategy. Next we sought to determine if our gene therapy strategy also works in a "humanized" mouse model carrying a CMT2D-patient-associated mutation ("delETAQ") in the mouse *Gars* gene (*Gars*$^{delETAQ/huEx8)}$). Here we show that scAAV9-delivered RNAi targeting delETAQ transcripts halts the progression of axon degeneration and reverses gross symptoms of neuropathy in post-symptomatic *Gars*$^{delETAQ/huEx8}$ mice. These exciting data not only further confirm the proof-of-principle of our therapeutic strategy, but suggests that our gene therapy has the capability to be translated into the clinic. Success with this allele-specific approach would provide a promising avenue for treatment of CMT2D and other dominantly inherited neuromuscular diseases.

977. Intravenous Injection of a Mito Targeted AAV9 Delivering Wild Type Human ATP6 Reversed Paralysis and Prevented Death in a Transgenic Mouse Model of Leigh Syndrome Caused by a T8993G Mutation in Subunit 6 of Mitochondrial ATP Synthase

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Introduction: A mouse model (A6) harboring human mutant ATP6 was created by microinjection of mito-targeted AAV2 carrying sc-HSP-*mutATP6FLAG+mcherry* into mouse zygotes. The transgenic A6 mice exhibit the most common features of leigh syndrome and neuropathy, ataxia, and retinitis pigmentosa (NARP) including premature death, mortality high to 78%, paralysis, vision loss, and seizures and involvement of multiple organs. Intravenous administration of mito-targeted AAV9 with wild type ATP6 into two groups at the age of three and eighteen month old A6 mice was evaluated. **Methods:** Mutant hATP6 gene was amplified and sequenced from mouse mitochondria DNA. Immunofluorescences and two dimensional native/SDS-PAGE western blot were performed to detect mutant hATP6 expression with Flag, mCherry and Porin or ATP5A antibodies. Twenty A6 mice were randomly classified into two groups at the age of 3 month old. 10μL of mito-targeted AAV9/ATP6 (1×10^{12}vg/ml) was administrated to ten of A6 mice respectively by orbital sinus injection, another ten of A6 mice without treatment as controls. Mouse vision and motor function was respectively assessed by Pattern ERG and rotarod at 6 and 12 month old. Body weight was evaluated at 6, 12 and 18 month old. Ten paralyzed and fourteen hunched A6 mice were respectively administrated the same dose. Body weight of paralyzed A6 mice was recorded daily. The paralyzed A6 mice with 20% of bodyweight loss were sacrificed. The ratio of heart to body of hunched A6 mice was evaluated. Lifespan of A6 mice with paralyzed and hunched was recorded. **Results:** Immunofluorescences showed the mutant hATP6 was expressed and co-localized with mitochondrial marker porin in A6 paralyzed brain and spinal cord. Two dimensional native/SDS-PAGE western blot suggested that the mutant hATP6 assembled into murine mitochondrial complex V in A6 liver mitochondria. The first group of A6 mice exhibited 100% survival after one and half year injection, for untreated A6 mouse only 30% survival. The weight of treated A6M mouse was not significantly changed at 6, 12 and 18 month old compared with wild type mice, however body weight of untreated A6 mice significantly dropped ($P<0.05$). Pattern ERG showed A6 mice had better visual function than untreated A6 mice ($P<0.01$). Rotarod showed after injection latency-to-fall time of the treated group A6 mice was significantly improved from 180 seconds to 100 seconds at a speed of 4 to 10 rpm compared to wild-type mice from 125 seconds to 5 seconds ($P<0.05$). The movement with paralysis hind limbs mice was significantly improved after two days of injection of mito-targeting AAV9/ATP6. Seizures, turning around and head tilt of treated A6M mice were observed to be normal for 6 to 12 months. Lifespan of A6M mice with severely hunched and paralysis was increased more than 60 days after the treatment compared to untreated A6M mice only 4 days ($P<0.01$). **Conclusions:** Intravenous injection of mito targeted AAV9 successfully significantly reduce A6 mortality, improved A6 visual and motor function and also increase the lifespan of paralyzed or hunched A6 mouse, suggesting it may also do so for children with Leigh Syndrome.

978. Systemic Administration of AAV-PHP.B in Cats Does Not Increase CNS Transduction over AAV9

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Efficient delivery of AAV vectors across the blood-brain barrier (BBB) remains a significant limitation for gene therapy for neurological diseases. In mice, AAV9 is capable of crossing the BBB after systemic administration and preferentially targets astrocytes in adult animals. AAV-PHP.B is a potent new CNS-tropic capsid derived from AAV9 by directed evolution of a 7-amino acid library inserted at position 588 of VP1. AAV-PHP.B transduces the adult mouse CNS at ~80-fold greater efficiency than AAV9, with remarkable neuronal tropism. Here we tested whether the increased ability of PHP.B to cross the BBB translates to larger species with a more complex CNS such as in cats, where numerous disease models are available and actively used in the development of gene therapies for neurological diseases. To address this key question about the translatability of AAV-PHP.B, we generated AAV vectors encoding a nuclear localized GFP under a ubiquitous CBA promoter and tested CNS transduction efficiency in young cats.

AAV9-nlsGFP or AAV-PHP.B-nlsGFP vectors were administered in a peripheral vein in 4-week old cats at 4E13 vg/kg. We observed overt signs of neurologic disease as early as 10 days post injection (proprioceptive ataxia and attenuation of the pupillary light response, or PLR) that became severe by 21 days, with marked neuronal loss in the cerebellum, brainstem and midbrain in the absence of perivascular cuffing, across the two different capsids. Following these results, the study was repeated using AAV vectors encoding standard GFP. Animals injected with AAV9-GFP or AAV-PHP.B-GFP were euthanized at 21 days post-injection showing only mild ataxia and PLR deficits at the experimental endpoint. Histological analysis of different CNS regions showed no appreciable difference in GFP expression between the two serotypes. Further vector biodistribution analysis of several brain regions as well as peripheral organs showed only a modest increase in vg copy number in animals injected with AAV-PHP.B. Altogether our data in a large animal model does not reproduce the obvious benefit of using AAV-PHP.B over AAV9 in mice. This is the first report testing this capsid in a gyrencephalic brain, though a recent publication has shown similar results in lissencephalic marmosets. Overall these studies show variable efficiencies of AAV capsids across species and suggest inherent differences in the BBB of rodents compared to larger species.

979. Correction of GM2 Gangliosidosis in Neonatal, Juvenile, and Adult Sandhoff Mice Using a Bicistronic Hexosaminidase Vector

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GM2 Gangliosidosis is a group of neurodegenerative lysosomal storage disorders characterized by a deficiency in the Hexosaminidase A enzyme (HexA). α- and β- subunits heterodimerize to form the HexA enzyme, which functionally catabolizes GM2 ganglioside (GM2). Deficiencies in the α- or β- subunit interrupts this catabolism, leading to an increase in GM2 storage, which in turn is toxic to the neurons of the central nervous system. Mutations in the *HEXA* and *HEXB* genes lead to mutations in α- and β-subunit, giving rise to Tay Sachs disease (TSD) and Sandhoff disease (SD), respectively. These diseases are lethal in humans by the age of 4 in the infantile form. In the current study, we investigate the use of the administration of bicistronic adeno-associated virus serotype 9 (AAV9) vectors containing either the human or murine Hex genes (*HEXA* and *HEXB*) at multiple ages in SD mice. We hypothesize that earlier intervention will produce more beneficial outcomes than delaying treatment until later in life. We also hypothesize that administration of the human and murine bicistronic vectors will produce similar beneficial outcomes, and these bicistronic vectors will show better outcomes compared to a vector containing murine *hexb* alone. Intravenous administration of the bicistronic vector was performed at 0, 2, 4, and 6 weeks in SD mice, where the dosage administered to the 0 week cohort was 2.5E14vg/kg, and 1.25E14vg/kg was given to the 2, 4, and 6 week cohorts. Half of the treated mice in each cohort were euthanized at 16 weeks, coinciding with the humane endpoint of the untreated vehicle controls. The other half of the treated animals were observed until reaching a humane endpoint. To date, a significant increase in survival was observed using either bicistronic vector at all ages of administration ($p<0.001$). The mice treated at 0, 2, 4, and 6 weeks are currently surviving at 46.6, 49, 44.5, and 41.6 weeks with the human bicistronic vector and 16, 49, 52, 47.8 weeks for murine bicistronic vector respectively, and the murine hexb treated mice at 0 and 6 weeks are currently surviving at 47 and 49 weeks respectively. Behaviourally, there was a significant improvement in performance of the treated SD mice at all ages of administration compared to untreated vehicle controls in the Open Field Test, Weight Test, and Rotarod. HexA enzyme activity levels were shown to increase post treatment in serum collected from the 6-week administration cohort. The bicistronic vector containing human transgenes elicited a small (nonsignificant) T cell response compared to the murine vector. Analysis of antibody response, brain hexosaminidase activity, GM2 storage, and vector biodistribution is pending.

Liver Monogenic Diseases: Genome Editing, AAV Vectors, and Cell Therapy

980. Targeted Integration of *MUT* into the *Albumin* Locus Using a Promoterless AAV Vector (Generide™) Confers a Hepatocellular Growth Advantage in Mice with Methylmalonic Acidemia

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Methylmalonic acidemia (MMA) is rare genetic disorder most typically caused by mutations in methylmalonyl-CoA mutase (*MUT*), a mitochondrial localized enzyme that occupies a critical role in the oxidative metabolism of isoleucine, valine and odd-chained fatty acids. Patients with MMA suffer from frequent, and potential lethal, episodes of metabolic instability, which accounts for the severe morbidity and early mortality observed in the patients. Liver transplantation is used as a treatment for some patients and can eliminate metabolic instability. While conventional adeno-associated viral (AAV) mediated gene delivery was highly effective in the treatment of neonatal mice with the most severe form of MMA, hepatocellular carcinoma, associated with AAV-mediated insertional mutagenesis in the *Rian* locus, has emerged as a long-term complication in aged mice. To minimize potential vector toxicity and increase the longevity of MUT expression after gene therapy, we have designed a promoterless AAV vector that utilizes homologous recombination to achieve site-specific gene addition of human *MUT* into the mouse albumin (*Alb*) locus, immediately upstream of mouse *Alb* stop codon. This new AAV

vector, AAV-Alb-2A-MUT, contains arms of homology flanking a 2A-peptide coding sequence proximal to the *MUT* gene, and generates *MUT* expression from the endogenous *Alb* promoter after integration. We have previously reported that AAV8-Alb-2A-MUT, delivered at a dose of 2.5E12 vg/pup at birth, reduced disease related metabolites, and increased growth in a hypomorphic murine model of MMA. In addition, we demonstrated that similar treatment with AAVDJ Alb-2A-MUT at a dose of 8.6E11 vg/pup rescued the neonatal lethal phenotype exhibited by complete *Mut* knock-out mice ($Mut^{-/-}$). We have now examined the durability of MUT expression in mice treated with AAV Alb-2A-MUT vectors, and assayed the expression of the MUT transgene using *in situ* hybridization with RNA scope detection 12-15 months post-gene delivery. Wild-type control mice, treated with either vector as neonates, had minimal evidence of long-term MUT expression, with ~1% or less of the hepatocytes staining positive after RNA scope. In stark contrast, the livers from the older treated MMA mice showed robust *MUT* expression in 10-20% of the total hepatocytes. The transduced cells appeared as distinct and widely dispersed clusters of hepatocytes that stained positive with the MUT RNA probe, and are consistent with a pattern of clonal expansion. In addition, the RNA scope studies revealed that the relative hepatic-transgene *MUT* expression was greater in treated MMA mice than treated wild-type mice. The aggregate findings suggest that there is a selective advantage for corrected hepatocytes in the murine models of MMA and predict that the potency of an AAV *ALB* GeneRide to treat MUT deficiency in humans could be enhanced. Additional studies will be needed to confirm our observations, which are the first to suggest a selective advantage after correction of hepatocytes in MMA.

981. Bezafibrate Enhances AAV Gene Therapy in Glycogen Storage Disease Type Ia

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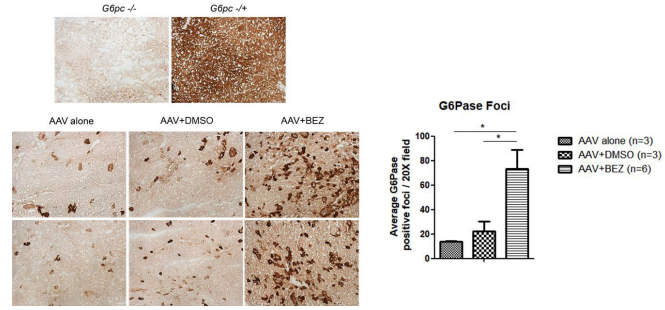
Glycogen storage disease type Ia (GSD Ia) is a rare inherited disease caused by mutations in the glucose-6-phosphatase (G6Pase) catalytic subunit gene (*G6PC*). Absence of G6Pase causes life-threatening hypoglycemia and long term complications due to the accumulations of metabolic intermediates including glycogen and triglycerides in the liver, kidney, and small intestines. We previously performed genome editing by delivering a G6Pase donor vector (AAV-RoG6P) together with a zinc finger nuclease containing vector (AAV-ZFN) to safely integrate the donor in the *ROSA26* locus. Genome editing with AAV-RoG6P and AAV-ZFN increased survival rate and transduction in hepatocytes, in comparison with the AAV-RoG6P alone group. Bezafibrate is well known as a pan-peroxisome proliferator-activated receptor (PPAR) agonist and used as a lipid-lowering agent to treat hyperlipidemia. Our preliminary data showed that bezafibrate treatment in GSD Ia mice lowered glycogen and triglycerides in liver. Based on previous results, we tested whether bezafibrate would enhance the efficiency of genome editing by normalizing hepatocellular abnormalities in GSD Ia. Here we present Bezafibrate treatment group showed increased survival rate after AAV administration and decreased liver size (liver/body mass, $p<0.05$), but not kidney size, in comparison with other control groups. In biochemical data, higher blood glucose level was detected ($p<0.05$) after 4 hours fasting and glycogen accumulation ($p<0.05$) was lower in association with higher G6Pase activity ($p<0.05$). Bezafibrate treatment was associated with higher *G6pc* mRNA levels ($p<0.01$) and higher G6Pase activity ($p<0.05$). Furthermore, bezafibrate-treated mice had a higher number of vector genomes for AAV-RoG6P in the liver ($p<0.05$), and their ZFN-mediated cutting was higher ($p<0.01$) as detected by the Surveyor assay, in comparison with other groups. Next, we investigated impaired molecular signaling in GSD Ia. The expression of PPARα, a master regulator of fatty acid β-oxidation, and of PPARγ, a lipid regulator signaling was increased in bezafibrate-treated mice. The expression of carbohydrate response element-binding protein (chREBP)-β was decreased in bezafibrate-treated mice, whereas sirtuin 1 (SIRT1), a deacetylase related to autophagy, was increased. LC3B-II, reflecting flux in autophagy, was fully restored in all vector treated mice. Therefore, bezafibrate improved the hepatic environment and increased the transduction efficiency of AAV vectors in liver, while higher expression of G6Pase corrected molecular signaling in GSD Ia. Taken together, bezafibrate shows promise not only as a treatment on GSD Ia, but also as a drug for increasing AAV transduction efficiency.



982. Conditional Disruption of Hepatic Carbamoyl Phosphate Synthetase 1 in Mice Results in Hyperammonemia without Orotic Aciduria and Can Be Corrected by *In Vivo* Liver-Directed Gene Therapy

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Carbamoyl phosphate synthetase 1 (CPS1) is a urea cycle enzyme that catalyzes the formation of carbamoyl phosphate using bicarbonate, ammonia and ATP. It is a large monomeric protein of 1462 amino acids. Bi-allelic mutations in the *CPS1* locus result in hyperammonemia without orotic aciduria, and the lack of early recognition of CPS1 deficiency typically results inencephalopathy, coma and early neonatal death. CPS1 deficiency has been described as the most severe of the urea cycle disorders and has an estimated incidence of 1 in 1.3 million births. Therapeutic interventions have limited efficacy and most patients develop long-term neurological sequelae. Using transgenic techniques, we generated a conditional *Cps1* knockout mouse. By loxP/Cre recombinase technology, deletion at the *Cps1* locus was

specifically achieved in the liver of adult transgenic animals using an adeno-associated viral vector expressing Cre recombinase under the liver-specific thyroxine binding globulin (TBG) promoter. The conditional knockout mice exhibited normal size and activity until the initiation of gene deletion by Cre recombinase expression. After about 14 days, animals began to develop sarcopenia as plasma ammonia began to rise. Within four weeks of AAV-Cre injection in these mice, all animals died with associated hyperammonemia and elevated glutamine without orotic aciduria; minimal detectable hepatic Cps1 protein was present. To assess the utility of this mouse model in the development of gene therapy for CPS1 deficiency, a helper-dependent adenoviral vector expressing murine *Cps1* (HDAd-*Cps1*) under control of the phosphoenolpyruvatecarboxykinase promoter was developed. A helper-dependent adenovirus was chosen based on its hepatotropic qualities and large capacity for DNA inserts required for *cps1* gene (4.5 kb cDNA) that remains episomal, reducing the concern for risk of malignancy. Liver-directed HDAd-mediated gene therapy resulted in extended survival, normalization of plasma ammonia and glutamine, and 13% of normal *Cps1* expression. Upon evaluating different viral doses of HDAd-*Cps1*, we demonstrate that 13% of native hepatic CPS1 gene expression is necessary to obtain survival in male animals to 30 days. A possible gender difference in survival suggests that female mice may require higher hepatic CPS1 expression. We conclude that this conditional murine model recapitulates the clinical and biochemical phenotype detected in human patients with CPS1 deficiency and will be useful to investigate ammonia-mediated neurotoxicity and for the development of cell- and gene-based therapeutic approaches.

983. Effective Treatment of a Novel Immunocompromised Conditional Arginase-Deficient Mouse Model with Human Hepatocyte Transplantation

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Arginase deficiency results from a loss of arginase 1 (A1), the final enzyme in the urea cycle, resulting in hyperargininemia. Arginase deficiency presents later in life compared to other urea cycle disorders typically beginning in late infancy to the second year with microcephaly, spasticity, seizures, growth retardation, episodes of hyperammonemia and failure to thrive. Currently, only marginally effective therapies are available and while new strategies, including preclinical application of gene therapy, have been applied and proven successful, limitations preclude their widespread use. Specifically, the extensive episomal loss of adeno-associated viral vectors (AAV) in rapidly dividing tissues such as neonatal liver, results in minimal residual hepatic AAV expression of A1 in adulthood and thus treated animals remain vulnerable to ammonia and at risk for hyperammonemia and death. Due to these limitations, other approaches, including cellular therapy and specifically, the transplantation, engraftment, and expansion of primary hepatocytes, deserve attention and an evaluation of their potential efficacy in treating arginase deficiency and other metabolic liver disorders that present early in life. However, such methods for treatment of urea cycle disorders in murine models have only been minimally explored. To explore the potential of this approach, we mated the conditional arginase deficient mouse (Arg-CKO) with a mouse deficient in fumarylacetoacetate hydrolase (FAH) and with Rag2 and IL2-Rγ mutations (FRG) to create the FRG-CKO mouse model. The Arg-CKO mouse featured a floxed A1 allowing for disruption of A1 activity by administration of tamoxifen or Cre-recombinase inducing hyperarginemia, hyperammonemia, behavioural abnormalities and death. The FRG mouse combined the ability to induce endogenous $FAH^{-/-}$ hepatotoxicity by withdrawal of the protective compound NTBC and T- and B-cell deficiencies by Rag2 and IL2-Rγ mutations to give a selective advantage to transplanted normal human hepatocytes. On day -1, a uroplasminogen-expressing adenoviral vector was administered intravenously and followed the next day with transplantation of $1x10^6$ primary human hepatocytes (PHH) (or vehicle alone) by intrasplenic injection. As the initial number of administered hepatocytes would be too low to prevent hepatotoxicity-induced mortality, NTBC cycling was performed to induce gradual endogenous hepatotoxicity and allow for the expansion and repopulation of the liver by the transplanted PHH. While all control mice died (n=8), all except two PHH-transplanted mice survived (n=14). Four months after PHH transplantation, $2x10^{11}$ genome copies of AAV-TBG-Cre recombinase was administered IV to disrupt endogenous hepatic arginase expression. While all control mice died within the first month (n=8), PHH-transplanted mice survived and remained healthy (n=10). Ammonia and amino acids were analysed in both control and transplanted groups before and after disruption of endogenous A1 expression and were well-controlled in the transplanted group while controls exhibited markedly abnormal levels. Ammonium challenging further demonstrated the durability and functionality of the human repopulated liver. Additional behavioural analysis demonstrated no statistical difference between PHH-transplanted and age-matched untreated control mice. Histologic examination showed repopulation of the murine liver with arginase-expressing human hepatocytes and any remaining murine hepatocytes were A1 negative. In conclusion, these studies demonstrate that human hepatocyte repopulation in the murine liver can result in effective treatment of arginase deficiency.

984. Successful *In Vivo* Editing of the OTC Locus in Primary Human Hepatocytes Xenografted into the FRG Mouse Liver

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The immense promise of liver-targeted gene therapy is in the early stages of realization, with progress underpinned by advances in AAV vector technology and the use of humanized preclinical models. While contemporary human clinical trials have exploited relatively tractable gene addition approaches, exciting developments in genome editing technology offer the prospect of locus-specific repair. To this end, we have chosen the human ornithine transcarbamylase (OTC) gene as a therapeutic target and developed functionally validated human locus-specific reagents for precise CRISPR/SaCas9-mediated cleavage and single nucleotide editing using AAV-mediated homology directed repair (HDR). This initial reagent development step was performed in the mouse liver following AAV/*piggyBac*-mediated transposition of the target human locus into mouse hepatocytes. These reagents, functionally validated for editing efficacy on the human OTC locus, were then experimentally evaluated *in vivo* on the native OTC locus in primary human hepatocytes xenografted into the FRG ($Fah^{-/-}Rag2^{-/-}IL2rg^{-/-}$) mouse liver. Using this model, we characterized the genome editing events in primary human hepatocytes after AAV-mediated delivery of the editing reagents pseudo-serotyped with the novel human hepatotropic capsid, NP59. We found that the locus-specific CRISPR/SaCas9 cutting efficiency in primary human hepatocytes was high (70% of OTC alleles), while the HDR frequency at the cut locus was relatively low, with 2% of alleles showing the expected editing of a single nucleotide. Most of the editing events observed were produced by a homology-independent mechanism, resulting in deletions (28.7%), and small (23.4%) and large insertions (17%). Interestingly, the large insertions resulted from integration of vector fragments at the target site. This high frequency of homology-independent *versus* homology-dependent events is likely to reflect, at least in part, limitations imposed by the cell cycle because HDR is inherently dependent on cellular proliferation. These data provide the first direct evidence of locus-specific single nucleotide editing in primary human hepatocytes *in vivo*, and also offer insight into the likely strategies that will be required to achieve efficient locus repair in tissues with a relatively quiescent mitotic status, such as the adult human liver. Based on these data we are currently evaluating editing strategies that exploit NHEJ, such as homology-independent targeted integration.

985. Successful CRISPR/Cas9-Mediated Gene Editing in Murine Phenylketonuria (PKU)

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Introduction Phenylketonuria due to phenylalanine hydroxylase (PAH) deficiency is among the most common human inborn errors of metabolism (IEM). Contemporary therapy relies upon lifelong restriction of dietary phenylalanine (Phe) intake, but the majority of PKU adults are unable to maintain blood Phe within the desired treatment range through dietary therapy alone. Novel treatments that do not strictly depend upon dietary intervention are strongly desired. Previous efforts have demonstrated the efficacy of AAV-mediated liver-directed gene addition strategies in the treatment of hyperphenylalaninemic Pah^{enu2} mice, a model of human PKU, but gradual loss of AAV episomes from liver in this model limit the duration of the treatment effect. Here we report our preliminary results on the use of CRISPR/Cas9-mediated gene editing to permanently correct the pathogenic mutation, partially restore liver PAH activity, and lower blood Phe in Pah^{enu2} mice. **Methods Animal Model:** The Pah^{enu2} mouse model harbors a c.835T>C missense mutation (p.F263S) in exon 7 of the *Pah* gene. Homozygous $Pah^{enu2/enu2}$ mice exhibit complete liver PAH deficiency and elevated Phe on normal mouse chow. **AAV vectors:** The requisite CRISPR/Cas9 reagents were delivered to $Pah^{enu2/enu2}$ mice in two separate recombinant AAV vectors. In one, SpCas9 protein was expressed under the transcriptional control of a strong liver-specific promoter. A second rAAV2/8 vector included 2 kb of wild type mouse genomic sequence flanking *Pah* exon 7 as a repair template but also expressed a validated exon 7-targeted CRISPR gRNA using a U6 promoter. **Animal Procedures:** Three $Pah^{enu2/enu2}$ pups were injected intravenously with the combined rAAV2/8 vectors via the facial vein at 3 days age followed by daily intraperitoneal injections with vanillin, a potent NHEJ inhibitor, for 7 days post AAV treatment. A second injection of combined rAAV2/8 vectors was administered at 5 weeks age, followed by an additional week of vanillin. Blood was obtained for Phe measurement at 5, 8, 9 and 11 weeks age and with euthanasia at 12 weeks. Liver PAH activity was measured using a radiometric assay. The presence of correctly gene edited alleles was detected through Sanger sequencing and quantified using a custom TaqMan genotyping assay. **Results** No adverse effects of treatment were seen. At euthanasia, gene correction was detected in 12.7, 15.8, and 40.3% of liver genomes, yielding restoration of liver PAH activity to 5.4, 8.4, and 24.8% wild type activity respectively. Serum Phe had decreased to 842, 393, and 883 μM by euthanasia, levels significantly lower than in untreated male $Pah^{enu2/enu2}$ mice (1647-2027 μM; $p = 0.002$). **Conclusion** A significant challenge impeding the translation of murine PKU AAV gene addition therapy to the clinic is temporary therapeutic efficacy due to the high therapeutic threshold, the lack of selective growth advantage for PAH+ hepatocytes, and the gradual loss of AAV vector genomes from liver. In this study, reversion of the genetic aberration underlying the Pah^{enu2} disease model has the potential to overcome all of these obstacles. CRISPR/Cas9-mediated gene correction is largely reliant on robust expression of the Cas9 enzyme as well as DNA double strand break repair through the HR pathway. In this small initial study, repeat dosing with our dual AAV vectors and concomitant treatment with an NHEJ inhibitor resulted in physiologically relevant levels of gene correction, partial restoration of liver PAH activity, and significantly decreased serum Phe. Studies in further mice are necessary to confirm these results along with a critical examination of the necessity for repeat dosing or vanillin treatment and assessment of off target effects. CRISPR/Cas9-mediated gene correction is a promising novel approach to the treatment of PKU and allied IEM.

986. Development and Characterization of a Humanized Porcine Model of Phenylketonuria

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Introduction: To create and characterize a humanized porcine model of phenylketonuria (PKU) utilizing TALEN-mediated gene editing. **Methods:** An R408W mutation targeting Exon 8 was introduced into the *PAH* gene of porcine fibroblasts with a repair oligo designed to humanize the mutation by introducing 5 SNPs native to the human sequence. Somatic-cell nuclear transfer was then used to create the first $PAH^{R408W/R408W}$ pigs. This novel large-animal model will allow for development of directly-translatable gene-editing constructs. Biochemical and histological analyses were performed to evaluate the presence and activity of PAH. **Results:** Three pregnancies resulted in six live-born $PAH^{R408W/R408W}$ piglets that were hand-reared on a combination of bovine colostrum/milk replacer and phenylalanine-free infant formula (Phenex-1). Plasma phenylalanine concentrations were elevated at birth (296.25 μM) and severe hyperphenylalaninemia (>2,000 μM) and neurologic abnormalities were observed by 36 hours in piglets consuming colostrum only. Treatment with Phenex-1 resulted in normalization of neurologic function, decrease in plasma phenylalanine, and lower concentrations of phenylalanine in brain cortex. Liver homogenates revealed no PAH enzyme activity in $PAH^{R408W/R408W}$ tissues compared to controls (0.07±0.11 vs. 90.96±18.85 nmol tyrosine/mg protein/hr, respectively) despite Western blot analysis showing similar levels of expression. Immunohistological analysis of liver tissue showed minimal and heterogenous PAH expression compared to controls. **Conclusion:** Preliminary data suggest that the R408W mutation in pigs results in the expression of nonfunctional PAH leading to complete PAH activity deficiency similar to what is seen in human PKU patients with the same mutation. Future work will include further characterization of the model as well as the development of translational gene editing techniques.

987. Anc80 and AAV8 Vectors Mediate Equivalent Long-Term Hepatic Correction of Methylmalonyl-CoA Mutase Deficiency in a Murine Model of Methymalonic Acidemia (MMA)

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Other than dietary and cofactor therapy, no alternative to organ transplantation exists for patients with isolated methylmalonic acidemia (MMA), a common and severe organic acidemia most frequently caused by mutations in the enzyme methylmalonyl-CoA mutase (MUT). *Mut* knock-out ($Mut^{-/-}$) mice replicate the phenotype of the most severe form of MMA and perish in the immediate newborn period. The introduction of a germ line transgene configured to express *Mut* in the skeletal muscle of $Mut^{-/-}$ mice has allowed the generation of mice, $Mut^{-/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$, that are rescued from lethality yet display severe biochemical perturbations, growth failure, and hepatopathy. $Mut^{-/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$ mice accurately mirror the severe childhood form of isolated MMA and provide a more physiologically relevant model to assay liver-directed gene therapy than neonatal $Mut^{-/-}$ pups. We have therefore used adult $Mut^{-/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$ mice to test the effects of systemic AAV gene therapy to mediate long-term hepatic expression of MUT. We compared a canonical hepatotrophic AAV serotype 8 vector configured to express the human *MUT* gene under the control of the alpha-1 antitrypsin promoter (AAV8-hAAT-MUT) to the same vector transgene pseudotyped with the novel capsid, Anc80 (Anc80-hAAT-MUT). Anc80 is an in silico-designed synthetic capsid and a putative ancestor of natural AAV serotypes, including AAV2, AAV8 and AAV9, with a reduced cross-reactivity with naturally occurring AAV serotypes. Adult female $Mut^{-/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$ mice and age-matched $Mut^{+/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$ littermates received $5x10^{12}$ GC/kg of either Anc80 or AAV8 vector (n=3 per group per vector) delivered by retro-orbital injection. In the MMA mice, the clinical and metabolic effects of gene therapy with both vectors were apparent by day 12 and manifested as statistically significant increases in weight, decreased metabolites (plasma methylmalonic acid and methylcitrate), and increased 1-^{13}C propionate oxidative capacity. The AAV treated animals experienced life-long maintenance of weight and metabolic stability, as evidenced by stable, but reduced, levels of circulating metabolites, and preserved 1-^{13}C propionate oxidative capacity. More than one year after gene therapy, all the AAV treated mice were sacrificed for biochemical and histological studies. In the treated $Mut^{-/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$ mice, MUT protein was detected in all hepatic extracts, near the level of $Mut^{+/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$ controls. AAV vector genomes ranged from 3-72 copies per cell. Gross dissection and further hepatic histology was without evidence of hepatocellular carcinoma or dysplasia in any mice that received AAV. LFTs were similar between controls and mutant groups with treated with either AAV8 or Anc80 vectors. Variable correction of the features of MMA liver disease was noted in the treated mutants, without correlation to relative hepatic MUT expression or metabolite levels. Further studies with RNA *in situ* hybridization in the livers from treated $Mut^{-/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$ mice revealed transduced cells that were scattered throughout the parenchyma and stained positive for *MUT*. In parallel, mice treated with lower doses of the same AAV vectors ($5x10^{11}$ GC/kg or $5x10^{10}$ GC/kg) were also studied in a similar fashion 1 year after treatment. Lower doses of AAV resulted in variable long-term correction, and fewer numbers of vector genomes in the liver. All vectors and doses induced the formation of anti-capsid antibodies. Our studies to date further demonstrate the functional equivalency of AAV8 and Anc80 vectors to mediate robust hepatic correction of Mut deficiency in MMA mice, with durability and lack of toxicity.

Cell Engineering and Clinical Trials

988. 3CAR: Gene Edited Anti-CD3 Chimeric Antigen Receptor T Cells

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T cells transduced to express chimeric antigen receptors (CARs) against B cell and myeloid antigen targets are being widely investigated. To date approaches to effectively target T cell malignancies have been hampered by 'T on T' cytotoxicity, a fundamental issue during the manufacture of therapies where T lineage antigens are targeted. Recent improvements in gene editing allow efficient disruption of such antigens and this has provided a route to generation of 3CAR, CD3-specific CAR T cells. We generated a lentiviral vector for delivery of a second generation CAR incorporating a codon optimised anti-CD3ε scfv (3CAR). Expression of endogenous cell surface CD3 was disrupted by editing the T cell receptor alpha chain locus in order to prevent expression and subsequent assembly of the multimeric TCR/CD3 complex. Consequentially, these cells escaped 3CAR cytotoxicity, and within 7 days of initiation, 52-65% of T cells expressed 3CAR. Importantly, the cultures 'self enriched' for T cells devoid of TCR /CD3, obviating the need for any further processing to deplete residual TCR+ T cells which comprised <0.2% of the product. Specific cytotoxicity has was demonstrated against CD3+ leukaemic T cell lines and primary T cells from healthy donors and subjects with T cell acute lymphoblastic leukemia (T-ALL).^{51}Cr- labelled TCR/CD3+ or TCR/CD3- Jurkat leukemia cells were cocultured with either 3CAR T cells or non-transduced control T cells. 3CAR+ cells mediated specific high level cytotoxicity of targets expressing TCR/CD3+, but not TCR/CD3- targets. Also single-cell flow-based assay GFP expressing TCR/CD3+ Jurkat cells were almost completely eliminated by 3CAR cells (0.8%), compared to untransduced effector controls (61%). Consistently, 3CAR T cells also mediated cytotoxic effects against primary CD3+ T cells purified from healthy donor peripheral blood mononuclear cells (PBMC) and loaded with CSFE. Only around 4% of these cells survived compared to 42% in control cultures, whereas TCR/CD3- populations were not eliminated by 3CAR cells, confirming specificity against CD3ε.To evaluate the function of 3CAR T cells against viable tissue bank samples from children with T cell acute lymphoblastic leukemia (T-ALL), flow cytometry verified CD3 and CD19 expression. In 6/6 T-ALL samples, 3CAR cells displayed highly specific cytotoxicity against CD3+ but not CD3-CD19+ cells. Furthermore, 3CAR T cells mediated potent anti-leukemic effects in a human:murine chimeric model. Clearance of TCR/CD3+ Jurkat T cells in 5/5 animals in 3CAR treated animals compared with proessive disease in control animals. Our findings, support the notion of a cellular immunotherapy strategy against T cell malignancy which could be adopted as a 'bridging' strategy to achieve leukaemic remission ahead of a conditioned allogeneic stem cell transplant.

989. Transposon-Modified T-Lymphocytes for Sustained Erythyopoietin Delivery *In Vivo*

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The development of a cell therapy platform for safe and long-term delivery of peptide hormones *in vivo* would be a significant advance for patients with a variety of hormonal deficiencies. In these studies we demonstrated the utility of antigen-specific T lymphocytes as a regulatable peptide delivery platform for sustained in vivo therapy. The non-viral *piggyBac* transposon system enabled stable genetic modification of both mouse and human antigen-specific T cells. Modification of murine cells with luciferase for *in vivo* imaging allowed us to visualize T cells after adoptive cell transfer (ACT). We used OT-1 T-cells that express a transgenic T cell receptor (TCR) for a peptide fragment derived from chicken ovalbumin (SIINFEKL) presented on H2-Kb MHC class I, and SIINFEKL as the vaccine antigen. We observed long-term T cell engraftment, persistence, and transgene expression enabling detection of modified cells up to 300 days after adoptive transfer of luciferase modified OT-1 T cells and a novel cell vaccination protocol. Vaccination augmented adoptive transfer of antigen-specific T cells which were transposon modified to express murine EPO (mEPO) and vaccination resulted in an increase of mEPO from a mean of 193 ng/ml to 4405 ng/ml 24 hours after adoptive transfer. Four weeks after ACT plasma mEPO concentration fell to a mean of 546 ng/ml. In order to determine if the plasma mEPO concentration could be further increased by the cellular vaccine we administered the vaccine on day 41 after ACT and observed a mean plasma mEPO concentration of 2312 ng/ml 24 hours after vaccination. Our adoptive transfer and vaccination strategy resulted in an elevation of hematocrit in WT mice for more than 20 weeks. We also demonstrated normalization of hematocrit in an adenine-induced nephrotoxicity anemia model. We extended our observations to human T cells demonstrating inducible EPO production from Epstein-Barr virus antigen-specific T-lymphocytes. Our results reveal antigen-specific T-lymphocytes to be an effective delivery platform for therapeutic molecules such as EPO *in vivo*, with important implications for other diseases that require peptide therapy.

990. Closed System Manufacturing of Expanded and Activated Gamma/Delta T Cells as Preemptive Immunotherapy in Haploidentical Hematopoietic Cell Transplantation: a Phase I Trial

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INTRODUCTION: Haploidentical hematopoietic cell transplantation (HAPLO HCT) with cyclophosphamide-induced activated T cell depletion (TCD) has shown enormous potential in patients with hematologic malignancies although applicability is limited

by a higher incidence of graft rejection and disease relapse. The addition of donor gamma-delta (γδ) T cells could constitute an effective prophylaxis against infection and relapse as they facilitate engraftment, exhibit strong graft-versus-leukemia effects, and do not initiate graft-versus-host disease. We report our recently developed closed-system cGMP compliant γδ T cells manufacturing process for a pivotal Phase I clinical trial in HAPLO HCT. METHODS: Four volunteer donor apheresis products were phenotyped, subjected to automated density gradient separation, and activated using a combination of Zoledronate and Interleukin-2 (ZOL/IL-2) in OpTimizer serum-free culture media with regular media exchanges. Following 14±3 days' culture in the Prodigy® bioreactor (Miltenyi Biotech; Bergisch Gladbach), αβ T cells were depleted (αβTCD) using biotinylated anti-αβ monoclonal antibody and streptavidin-coated ferromagnetic microspheres. Products were analyzed for expansion, phenotype, viability, potency, and microbiologic contamination using standard clinical laboratory procedures. RESULTS: The % γδ T cells increased from 3.6±3.8 (range 1.2 to 9.2) to 53.8±21.2 (range 36.0 to 84.0) prior to αβTCD and 89.0±15.1 (range 66.5 to 90.0) in the final product. As expected, the γδ T cells were overwhelmingly Vγ9Vδ2+CD4-CD8-CD28+, incrementally expressed CD56 and PD-1, and were effector and effector/memory phenotype. NK cells also comprised 1.4% to 32.0% of the αβTCD product and total αβ T cell total dose was $<1.0 \times 10^5$/kg assuming a 70kg recipient although substantial loss of γδ T cells also occurred (69% to 78%). Final cell viability ranged from 62% to 67%. When extrapolated to a 2-volume apheresis collection all products met dose level of 3×10^6 cells/kg, our predetermined criteria for initiation of the trial. Cytotoxicity against K562 cells showed variability as expected, 52.7±22.4 (range 33.0 to 84.0) but passed predetermined specifications at an effector to target (E:T) ratio of 20:1. Gram stain, 14-day bacterial culture, endotoxin and mycoplasma were negative for organisms in all processing runs. CONCLUSIONS: Automated, cGMP compliant γδ T cells expansion is feasible, reproducible, and sufficiently reliable for advancement into clinical trials. Remaining challenges in process development include excessive loss of γδ T cells in the αβTCD step and scaling to higher doses across all potential patient weights, however, the automation of closed-system GMP γδ T cells expansion should widen acceptance and applicability of this procedure in HAPLO HCT and other settings.

991. Phase 1 Feasibility and Safety Study of Lentiviral Gene-Modified Epidermal Grafts for Netherton Syndrome

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Netherton syndrome (NS) is a rare autosomal recessive skin disease caused by null mutations in *SPINK5*, resulting in loss of function of its encoded protein Lympho-epithelial Kazal-type-related inhibitor (LEKTI). Patients with NS have congenital ichthyosiform erythroderma, trichorrhexis invaginata and atopic manifestations. There is high mortality and morbidity in the first year of life due to hypernatraemic dehydration secondary to severe water loss through the defective skin barrier. There is no curative treatment but emollients may improve compromised skin. We report a first-in-human gene therapy safety study for NS using lentiviral delivery of codon-optimised *SPINK5* to primary NS keratinocytes to generate gene-modified autologous epithelial skin sheets. A codon-optimised *SPINK5* gene was linked to a 750bp promoter element for human involucrin to support compartment-specific expression of *SPINK5* in the upper epidermis. Procedures were established for the production of lentiviral-engineered epithelial skin sheets under Good Manufacturing Practice conditions. Five NS adults were enrolled and a 6mm skin biopsy from each patient was taken for isolating keratinocytes including keratinocyte stem cells. Keratinocytes were cultured using irradiated 3T3 feeder cell lines and specialised media conditions, and transduced at MOI 20. All transduced cells exhibited over-expression of the *SPINK5*-coded protein, LEKTI, and were found to harbour viral copies of between 1.8 to 11.6/cell. Full-length LEKTI was detected using immunoblotting and positive LEKTI expression in modified epithelial sheets was confirmed using *in situ* immunostaining. Epithelial sheets were successfully generated in 3 out of 5 subjects and one went on to receive a gene-modified graft (4x5cm) on the anterolateral aspect of left thigh following surgical de-epidermalisation. The grafted patient had no serious adverse events over the 12-month follow-up with negative ELISPOT pre- and post-gene therapy. Vector copy and LEKTI expression were transiently detectable up to 3 months in serial biopsy samples, indicating probable loss of critical long-lived keratinocyte stem cell populations over time. The overall approach requires additional strategies to identify, target and foster engraftment of gene modified stem cell populations for durable efficacy.

992. Production of Good Manufacturing Practice Compliant Gene Engineered Autologous Fibroblasts for Recessive Dystrophic Epidermolysis Bullosa

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Recessive dystrophic epidermolysis bullosa (RDEB) is one of the most severe forms of epidermolysis bullosa. It is a debilitating genodermatosis caused by loss-of-function mutations in *COL7A1*,

which encodes type VII collagen (C7), a protein important for anchoring fibril formation at the dermal-epidermal junction (DEJ). We report a GMP compliant protocol for the generation of *COL7A1* gene-engineered autologous fibroblasts for a Phase I clinical trial in RDEB patients. A compliant stock of 3rd generation self inactivating (SIN) lentiviral (LV) vector encoding a codon optimized *COL7A1* transgene linked to the human PGK promoter (LV-*coCOL7A1*) was produced and subjected to detailed release characterization, including screening for replication competent lentivirus. Primary fibroblasts isolated from 6 mm punch skin biopsies were obtained from subjects with RDEB. Cells were expanded for 18 - 28 days, until the desired number of cells were reached (~2x10^6), and transduced with LV-*coCOL7A1* at MOI 5. Once the transduction was successfully completed, gene engineered fibroblasts were further propagated for 3 to 4 weeks in T-75 culture flasks, then harvested and cryopreserved. Sterility testing including 7 day bacterial cultures and PCR based screening for mycoplasma were carried out. A small proportion of the gene engineered cells were used for the assessment of viral copy number (0.17 - 0.56 copies per cell) and for the confirmation of C7 expression by flow cytometry (3.5 to 12 % C7 positive cells). Furthermore, a full-length C7 band on western blot analysis and over expression of C7 in *In-situ* immunostaining were confirmed in the gene engineered fibroblasts. Three doses of the investigational medicinal product (IMP) comprising 0.8 - 1.2 x10^6 cells suspended in 0.25mL of 0.9% saline were assembled in 1mL syringes after thawing and washing the cells on the day of injection. The products were all released for a Phase 1 safety study of lentiviral gene engineered fibroblasts expressing C7 in RDEB.

993. Efficient Clinical Scale CRISPR/Cas9-Mediated Editing of Plerixafor-Mobilized Hematopoietic Stem and Progenitor CD34$^+$ Cells for Treatment of Sickle Cell Disease

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Sickle cell disease (SCD) results from a recessive autosomal mutation in the human β-globin gene leading to anemia, painful sickle cell crisis, and progressive organ damage. Allogeneic hematopoietic stem cell transplantation (HSCT) is currently the only curative treatment but limited to patients with matched sibling donors. Reactivation of fetal globin expression by genome-editing of hematopoietic stem and progenitor cells (HSPCs) to knock down γ-globin repressors, such as BCL11A, has been explored as an autologous HSCT therapy for SCD. We describe here the development of a semi-closed, clinical scale production process for CRISPR/Cas9-mediated editing of human HSPC CD34+ cells. To simulate the production process for the proposed product, we used leukopaks collected from healthy donors after stem cell mobilization by plerixafor as the source material. We incorporated several GMP-compliant, automated, closed systems into the process, including the Miltenyi CliniMACs Prodigy for HSPC CD34+ enrichment, and the MaxCyte GT electroporator for transiently introducing CRISPR/Cas9 reagents into HSPCs to mediate the genome-editing. Our process leads to highly efficient allele-editing of BCL11A by the lead guide RNA candidate without detectable compromise of cell viability. This high level of the editing could result in most of the population of cells edited bi-allelically at the targeted locus, as indicated by genotyping analysis of erythroid colonies derived from edited HSPCs. Moreover, when induced to differentiate into myeloid or erythroid lineage *in vitro*, the edited HSPCs displayed comparable growth and cell characteristics to unedited controls, while maintaining the editing level throughout the differentiation process. Importantly, we observed a substantial increase in population with detectable γ-globin expression in edited erythroid progeny over the un-edited controls. Together, these results demonstrate efficient clinical scale editing of BCL11A in plerixafor-mobilized HSPC CD34+ cells and support the clinical development of CRISPR/Cas9 editing of BCL11A as autologous HSCT therapy for SCD.

994. Genome-wide Integration Profile of a Lentiviral Vector Carrying the Human *FAH* Gene in Human Hepatocytes

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Introduction: Lentiviral (LV) vectors can deliver large amounts of genetic information into dividing and non-dividing host cells with high efficiency, generating stable expression of the transgene. These characteristics make LV vectors an excellent option for gene delivery and gene editing; however, potential for insertional mutagenesis remains a concern. This study investigates the whole genome profile of LV integration to better characterize and evaluate its genotoxicity. **Methods:** The LV vector used in this study was constructed carrying human *FAH* gene expressed under the hepatic control region enhancer/human alpha-1 antitrypsin promoter complex (HCR-hAAT). Human hepatocytes were transduced with LV vector at 2000 MOI, 4°C for 90 minutes and 37°C for 30 min. Genomic DNA was extracted at 72 h from the transduced hepatocytes. Standard PCR using LV-specific primers was used to confirm genome integration. The diverse libraries of LV integration sites were generated through ligation-mediated PCR (LM-PCR) amplification. The amplicon pools were sequenced through next generation sequencing (NGS). The genomic location of each virus-host junction was mapped using BWA MEM. **Results:** A total of 77.6 million integration sites were detected with 1.2 million being unique. Mapping of all integration sites to human chromosomes shows most hotspot integration sites correspond to less condensed, more structurally accessible regions of the genome. Additionally, virus integration frequency was correlated to gene density in each chromosome and relative expression level of each gene. Lentivirus preferentially integrated in transcription unit, especially introns, and much less frequently in exons and other non-coding regions. Dividing LV integration into quartiles of the coding region we found no preference to a particular quartile. Lentivirus also demonstrated a preference for integration beyond 20kb to transcript start sites (TSS) but not in the promoter region. After normalization for all CpG islands in the human genome, CpG islands were not preferred for lentivirus integration suggesting a low frequency of promoter disruption. When integration frequency within tumorigenesis-related genes was normalized for all genes in the human genome, we observed no preference for integration into tumor-associated genes, with integrations typically occurred in

the intronic region with no preference to the promoter region. Finally, we show for the first time that sequence homology may play role for integration preference as overwhelming integration events occurred at the *FAH* locus and *SERPINA1* gene, which has homology with HCR-hAAT. **Conclusions:** Evaluation of 77.6M LV integration sites provided a meaningful data set for genotoxic evaluation of lentivirus integration. Whole genome integration mapping shows most hot spots occurred in less condensed regions in the genome. LV integration sites were highly related with gene density in the human genome, and typically within transcribed regions with a preference for introns. CpG islands were not favored by lentivirus for integration and integration was positively correlated to gene expression level. We also showed there was no significant integration preference towards tumorigenesis related genes when compared to the whole genome. LV-huFAH integrated frequently into the *FAH* gene in human hepatocytes, indicating the possibility that sequence homology may affect integration site selection.

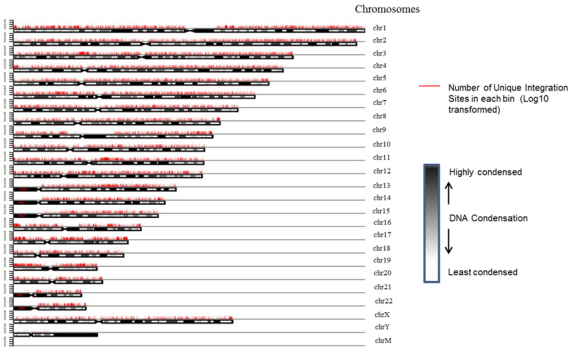

Figure. Whole genome view of LV-huFAH integration in Human Hepatocytes

Each chromosome in the figure is divided into "bins" of 10kb and the red vertical bars indicate the log transformed number of unique integration sites within each bin. The "hotspot" integration sites can be seen in regions where there is a dense grouping of the red vertical bars. Most "hotspot" regions correspond to lighter band regions of the chromosomes indicating the presence of integration sites in less condensed (i.e. more accessible) regions of the genome.

995. Changing Cell Manufacturing Processes during Clinical Trials: A Success Story

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Tisagenlecleucel is the first approved CD19-directed genetically modified autologous T cell immunotherapy indicated for treatment of patients up to 25 years of age with B-cell precursor acute lymphoblastic leukemia (B-ALL) that is refractory or in second or later relapse. CAR-T cell processing technology for tisagenlecleucel was validated and scaled to ensure widespread distribution to patients in large global trials and in the commercial setting. Tisagenlecleucel manufactured in centralized facilities (Morris Plains, NJ, USA and Fraunhofer Institute, Leipzig, Germany) has been used successfully in worldwide multicenter trials for treatment of relapsed/refractory pediatric B-ALL (ELIANA; NCT02435849) and adult diffuse large B-cell lymphoma (JULIET; NCT02445248). Here we describe the continuous process improvements made to the tisagenlecleucel manufacturing process over time to meet demand while maintaining or improving product quality. Reversible logistical challenges in manufacturing earlier in multicenter clinical trials led to higher than expected dropouts between enrollment and infusion. Enhancements were made to improve manufacturing capacity, process robustness, process success rate, and product quality as well as to reduce throughput time. Cryopreserved leukapheresis product was selected as the starting material over fresh product to ensure flexibility for patient management during centralized manufacturing. T-cell enrichment pathways for incoming leukapheresis material were further optimized based on clinical trial experience and now comprise a single improved pathway (flow-through antibody-based selection technique) to be used for a range of incoming cell populations. Staffing was also optimized for receipt of increased incoming volume of samples with a corresponding increase in clean-room capacity. Rate-limiting steps in the manufacturing process were optimized, including reduced use of human serum to overcome supply limitations and earlier release of product based on validation of a more rapid sterility assay. Areas of focus to enhance process control and consistency included closing process steps through customized consumable and equipment solutions to enhance sterility assurances, replacing some manual processes with automation to ensure consistent reproducibility, analytical method validation, and implementing more robust/faster analytical methods. An automated electronic system was introduced for order management and to track chain of identity. Shipping logistics were also evaluated including the cryoshippers (design of enclosed dewars [flask charged with liquid nitrogen] and temperature control mechanisms) and couriers used. Continuous process improvements in manufacturing during ongoing clinical trials resulted in a consistent and optimized process for tisagenlecleucel, with the goal of achieving a 22-day manufacturing cycle and a very high manufacturing success rate in the commercial setting. These changes were introduced during the course of the clinical development by incremental comparability protocols to ensure that each patient would receive product of consistent quality. Consistent product quality during manufacturing has been demonstrated by extensive product testing, including assessment of product T cell populations, vector copies per cell, CAR functionality, and absence of replication-competent lentivirus. Additionally, positive clinical outcomes were observed across the allowable range of product quality attributes in the global clinical trials. In summary, considerable experience has been accrued in manufacturing of autologous CAR-T cells in global, multicenter trials and has led to the development of a consistent and robust commercial manufacturing process for tisagenlecleucel, through continuous evaluation and improvements.

Immunological Aspects of Gene Therapy and Vaccines

996. FVIII Expression Driven by Its Native Promoter by Lentiviral Vectors Allowed Sustained Long-Term FVIII Expression Blunting Immune Responses in Hemophilic Mice

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AIM: A possible strategy to overcome inhibitors formation is represented by the restriction of FVIII expression in specific and tolerogenic cell type, preferably the natural FVIII-secreting cells. For this purpose, we studied the activity of F8 promoter (pF8) sequence to drive transgene expression in LV to verify the feasibility of expressing FVIII under its natural promoter for gene therapy approaches. Methods: Analysis of FANTOM5 collection of human libraries was performed using the Zenbu browser genomic tool. To dissect transcription start sites (TSSs) usage in the expression of F8, we interrogated FANTOM5 expression data comprising almost 2000 human samples, covering cell lines, primary cells and tissues. In silico analysis to predict transcriptional factor (TF) consensus sequences on pF8 was performed by the softweare PROMO 3.0. Several form of F8 promoters were cloned in LV carrying GFP or FVIII as transgene. GFP distribution was analyzed by FACS and immunofluorescence in cells and tissues. We injected LV.pF8.hFVIII in C57/BL6 and 129-BL6 hemophilic mice and aPTT and ELISA assays performed to measure FVIII activity or inhibitors formation. Immunocompetent hemophilic mice (B6/129) were tail vein injected with 109 TU LV.pF8. FVIII-BDD. Depletion of regulatory T cells (Tregs) in mice by anti-CD25 injection. Results: FANTOM5 data confirmed the usage of previously described F8 TSS, which promotes the transcription of the annotated reference sequence (NM_000132). One of them resulted the most highly used in FANTOM5 samples, inducing the expression of F8 in 151 libraries. In silico analysis of Transcriptional Factors (TF) consensus sequences predicted the presence of several myeloid-specific TF, in addition to hepatocytes- and endothelial-specific TF. To verify the prediction we injected several LV.pF8.GFP constructs in C57Bl/6 mice, GFP expression was restricted to liver sinusoidal endothelial (LSEC). Instead, in spleen and bone marrow, GFP was in myeloid cells. We injected LV.pF8.hFVIII in hemophilic mice and aPTT assay demonstrated FVIII activity in therapeutic range (up to 12% of normal FVIII activity) without antibodies formation up to 1 year and blood loss reduction in gene-corrected hemophilic mice. Moreover, LV pF8.hFVIII injection in previously FVIII-immunized HA mice resulted in therapeutic FVIII activity and complete reversion of inhibitor titers. Furthermore, immunization with FVIII failed to induce antibodies in all treated mice without affecting FVIII activity Bleeding challenge performed one year after injection showed that blood loss was significantly reduced in all treated HA mice thus confirming the achievement of the phenotypic correction. Using more active forms of FVIII cDNA we improved FVIII activity levels in HA mice up to 25% of normal FVIII activity. To elucidate the role of pF8 in tolerance induction to FVIII after in vivo LV.pF8.FVIII-BDD delivery we proceeded with depletion of regulatory T cells (Tregs) in mice . FVIII activity in plasma of all treated mice reached 10-11% and remained stable up to 10 weeks. Eleven weeks later we depleted Tregs in mice by anti-CD25 injection and 3 weeks after Tregs depletion FVIII started to decrease with concomitant formation of anti-FVIII antibodies only in mice in which Tregs were depleted. Conclusion: Expressing FVIII under its promoter we were able to reach therapeutic sustained levels of FVIII in HA mice without immune response, the expression level was higher than with other endothelial-specific promoters. Our data suggest that FVIII expression using the pF8 is mainly secreted by sinusoidal endothelial cells in the liver and Tregs are involved in tolerance induction to FVIII after gene transfer.

997. *In Vivo* Activation of Dendritic Cells by AAV Vectors and NK Cell-Independent Cross-Priming of CD8$^+$ T Cells

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Recent clinical trials have shown that AAV-mediated gene therapy can cure monogenic disorders such as hemophilia. However, activation of CD8$^+$ T cells specific for the AAV capsid has hampered clinical progress, and the initial steps of CD8$^+$ T cell priming in response to AAV remain undefined. Our previously published work established a requirement for two dendritic cell subsets, plasmacytoid dendritic cells (pDCs) and conventional DCs (cDCs), each with critical but discrete roles throughout the early priming phase (*Blood* 129:3184). Specifically, TLR9 sensing was required in pDCs while cDCs were critical for cross-presentation of AAV vector-derived antigen. Type I interferon (T1 IFN) is heavily produced downstream of TLR9 in pDCs and is an important mediator of anti-viral immunity. Indeed, blocking T1 IFN signaling abrogated cross-presentation by cDCs and anti-capsid CD8$^+$ T cell priming, underscoring the importance of T1 IFNs as an innate immune activation signal. We therefore propose a model where innate sensing of AAV occurs through TLR9 in pDCs results in production of T1 IFNs that license cDCs *in trans* to permit anti-capsid CD8$^+$ T cell priming. T1 IFNs can modulate CD8$^+$ T cell priming by promoting cDC migration, cross-presentation and expression of co-stimulatory markers. Therefore, we sought to determine how AAV-elicited T1 IFN augments cDC activation. To test the activation status of cDCs in response to AAV we measured surface expression of MHC class II and co-stimulatory molecules from both the B7 family and TNFR superfamily on pDCs, and two subsets of cDCs: CD8α$^+$ cDC1s and CD11b$^+$ cDC2s. C57BL/6 mice were injected i.m. with either AAV2, PBS, or CpG DNA. Spleens and inguinal lymph nodes (ingLNs) were analyzed by flow cytometry 48hrs later. Consistent with previous reports, expression levels of CD86 and MHC II were not upregulated, and we observed no change in expression levels of CD40, OX40L, and ICOSL. However, in the ingLNs of AAV treated mice, we found that AAV induced elevated surface expression of 4-1BBL in cDC1s but not cDC2s or pDCs. In addition, we found an overall increased frequency of DCs and an increase in surface expression of PDCA-1 on cDCs, which is known to be induced by T1 IFN signaling. Together, these data suggest that following AAV treatment, T1 IFN signaling in cDCs promotes their migration and coordinately induces expression of

select costimulatory molecules in a cell specific manner. T1 IFNs also potently activate natural killer (NK) cells, which produce inflammatory cytokines that can enhance cDC activation and the development of effector functions in primed $CD8^+$ T cells. Thus, we tested for a requirement of NK cells in anti-capsid $CD8^+$ T cell priming. NK cells were depleted *in vivo* with αNK1.1 (PK136) monoclonal antibody 1 day prior to AAV injection in C57BL/6 mice. Mice received $1x10^{11}$ vg of a modified AAV2 capsid that contains the peptide sequence SIINFEKL, so that capsid-specific $CD8^+$ T cells can be identified using H2-K^b-SIINFEKL tetramer. We observed no significant difference in $tetramer^+$ $CD8^+$ T cells in the blood at 7, 8, and 10 days p.i. compared to isotype control (n=5/group), suggesting that NK cells are not required for $CD8^+$ T cell priming. Additionally, an *in vivo* killing assay revealed that anti-capsid $CD8^+$ T cells primed in the absence of NK cells were functional. Therefore, anti-capsid $CD8^+$ T cell priming and development of effector functions is independent of NK cell function. Instead, we propose a direct effect of T1 IFN on cDC to acquire the capacity to cross-present capsid antigen and prime $CD8^+$ T cells.

998. Modeling and Modulating Anti-Transgene Immune Response in *Ex-Vivo* Gene Therapy for Mucopolysaccharidosis Type-I (MPS-I)

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Gene therapy is a promising therapeutic approach for genetic disorders, but its clinical efficacy in the context of immune competent patients may be threatened by undesired anti-transgene immune responses. Mucopolysaccharidosis type-I (MPS-I) is a lysosomal storage disease caused by the deficiency of alpha-L-iduronidase (IDUA) enzyme activity. The available treatments are enzyme-replacement therapy (ERT) and allogeneic hematopoietic stem cell (HSC) transplantation. An alternative therapeutic option is *ex-vivo* HSC gene therapy with a lentiviral vector encoding for IDUA (LV-IDUA), and preclinical studies performed in naïve MPS-I mice demonstrated its efficacy in the absence of pre-existing immunity against the enzyme, thus paving the way towards clinical application. However, several MPS-I patients undergoing ERT develop anti-IDUA IgG, thus suggesting the presence of an IDUA specific immune response, which may jeopardize treatment efficacy. To study the impact of pre-existing anti-IDUA immunity on gene corrected HSC engraftment, we developed an experimentally induced anti-IDUA immunity model. We injected MPS-I mice with IDUA and adjuvant and showed a consistent induction of anti-IDUA IgG and cytotoxic $CD8^+$ T cells. Immunized MPS-I mice were lethally irradiated and transplanted with LV-IDUA transduced HSC. After hematopoietic reconstitution, mice were sacrificed to quantify the transduced cells engraftment and the residual cellular and humoral anti-IDUA immunity. We showed that engraftment inversely correlated with the pre-existing anti-IDUA response: all IDUA-immunized mice cleared the IDUA-transduced and transplanted cells, but not control-transduced HSC, thus suggesting an antigen-specific mechanism of rejection. Passive transfer of anti-IDUA IgG in naïve MPS-I mice before the IDUA-transduced HSC transplantation demonstrated that anti-IDUA IgG do not affect engraftment of gene corrected cells. On the contrary, treatment of immunized MPS-I mice with T cell-depleting agents partially rescued engraftment of IDUA-corrected cells in IDUA-immunized animals. These data suggest a role for cellular immunity in transduced cells clearance. Overall these findings demonstrate, for the first time, the relevance of pre-existing anti-transgene immunity on *ex-vivo* gene therapy and illustrate the potential need to adapt the immunosuppressive component of the conditioning regimen in future gene therapy trials for MPS-I.

999. Review of CSF and Peripheral Immune Responses Following Intrathecal Gene Transfer for Giant Axonal Neuropathy

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GAN is a rare childhood onset neurodegenerative disorder of the peripheral and central nervous system. Recessive *GAN* mutations cause dysfunction of gigaxonin, a cytoskeletal regulatory protein, leading to progressive sensorimotor and optic neuropathy, CNS involvement and respiratory failure. Independent ambulation is lost by 10 years of age and death ensues by the 2nd to 3rd decade of life. We are conducting an intrathecal (IT) AAV9 mediated gene transfer trial for GAN (NCT02362438). Since this is the first-in-human application of intrathecal AAV9 for any indication, the safety and immunologic implications of IT gene transfer reported here should be relevant to gene therapy as a field.Eight cross-reactive immunological material-positive (CRIM (+)) GAN patients have been dosed at three dose levels (ranging from $3.5x10^{13}$ vg to $1.8x10^{14}$ vg) with scAAV9-JeT-GAN. Patients are pretreated with IV methylprednisolone, followed by transient oral prednisone. Two CRIM (-) or 'null' mutation patient have been injected at two dose levels ($1.2x10^{14}$ vg and $1.8x10^{14}$ vg) with additional specially adapted immunosuppression to minimize a the risk for an adaptive anti-transgene response. Clinical safety is assessed by neurologic exam, neuroimaging, serum and CSF studies,

EKG and Echocardiogram. Preexisting NAb titers are measured in serum and CSF and are followed after gene transfer. In addition, interferon-γ ELISpot analysis (to GAN and AAV9 epitopes), CSF and peripheral cytokine analysis and white blood cell flow cytometry (phenotyping) are performed at various time points. 30% of patients had positive preexisting serum AAV9 NAb titers but none had positive NAb titers in CSF at baseline. Serum and CSF AAV9 NAb levels rise following IT gene transfer. ELISpot analysis shows a T cell response to AAV9 but not to gigaxonin. A transient, dose-dependent and steroid-responsive clinically asymptomatic CSF pleocytosis was observed, without change on exam, imaging, or serum chemistries. This phase I IT AAV9 mediated gene transfer study for GAN is establishing proof of principle for this novel route of administration, while establishing safety, dose limitations, and elucidating immunological implications. This is an updated analysis presenting additional data from now ten patients with up to two year post-gene transfer follow up.

1000. Reversal of Multiple Sclerosis in Multiple Mice Models

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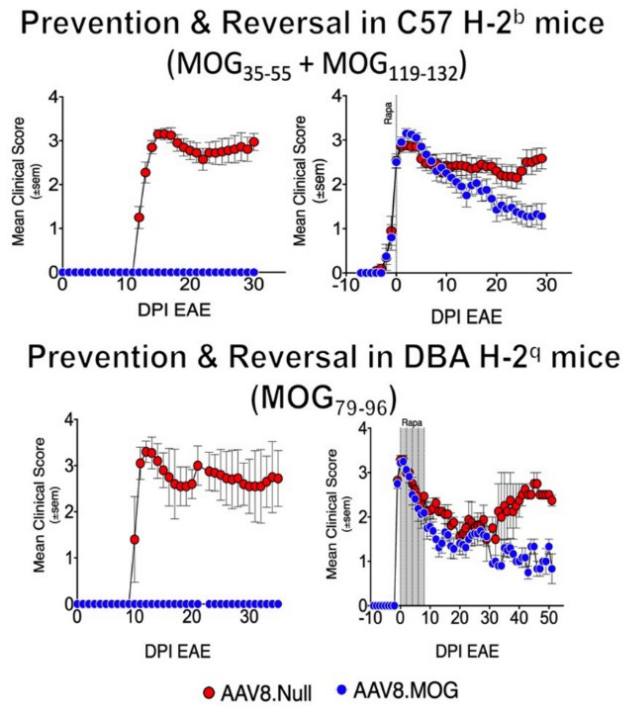
Multiple Sclerosis (MS) is a complex, inflammatory disease of the CNS. The treatment of MS is complicated by the fact that the encephalogenic epitopes remain unknown and can vary between patients as HLA classes change. The most effective treatment for this disease would be capable of treating disease, regardless of specific epitope. While the etiology of MS is unclear, there is significant evidence that a break in immune tolerance is a critical first step. The use of cell-based immunotherapies represents an attractive option for inducing or restoring immune-tolerance which has been postulated as being able to reverse MS. Regulatory T cell (Tregs) play a key role in both induction and maintenance of tolerance. Many strategies focus on harvesting and expanding autologous Tregs and then re-infusing them into patients. Unfortunately, the development of a large scale *in vitro* method for the reliable expansion of Tregs sufficient for treatment, has not been achieved. Further, most *in vitro* methods rely on the expansion and use of polyclonal Tregs, though antigen (Ag) specific Tregs are more effective. To overcome these issues, we have developed a clinically relevant, *in vivo* approach for inducing Ag-specific Tregs, via liver directed AAV gene transfer. Recently, we showed that AAV.MOG is capable of preventing EAE induced by MOG_{35-55}. More importantly, (Mol.Ther. Vol.26, 173-83). AAV.MOG immunotherapy is capable of completely reversing mild thru severe EAE, rescuing mice with advanced paralysis. Now, we demonstrate that our vector system has the ability to be effective against multiple immunoreactive MOG peptides in mice with differing MHC haplotypes. Making the therapy universally applicable regardless of antigenic epitope and strain specific genetics. First, we tested the ability of our vector to prevent and reverse disease induced with multiple epitopes (MOG_{35-55} & $MOG_{119-132}$) in C57 (H-2^b) mice. In prevention studies, control mice developed severe EAE, while treated mice showed no signs of disease. In reversal studies, the immunotherapy resulted in a significant reduction in disease severity in treated mice as compared to controls. Next, we investigated the ability of AAV.MOG to prevent disease induced by full-length MOG, which is capable of producing multiple known and unknown encephalogenic epitopes, in C57 mice. Again, control mice developed severe EAE while treated mice remained symptom free. Lastly, we investigated the ability of our treatment to be effective in mice of a different genetic background. In these studies, we induced EAE in DBA-1 mice (H-2^q) using MOG_{79-96}. As before, treatment conferred protection as control mice developed severe EAE and treated mice remained symptom free. Furthermore, when AAV.MOG immunotherapy was administered therapeutically, disease severity in treated mice was again significantly reduced as compared to controls. Histological evaluation revealed that treatment results in reduced inflammation and demyelination of the CNS, with no serological signs of liver damage. In sum, we have developed a viable and universal i*n vivo* immunotherapy that reverses paralysis in multiple murine models of MS that is independent of MHC haplotype or inducing protein antigen. The application of this approach as a clinical therapy for treating MS and other human autoimmune diseases warrants further investigation.



1001. Engineering AAV Vectors to Evade Innate Immune and Inflammatory Responses

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Many new therapeutic modalities including gene therapy rely on nucleic acid-based systems. However, nucleic acids can trigger host immune responses in vivo that hinder efficacy and reduce safety and tolerability. For example, it has been shown that the DNA genome of the viral vector adeno-associated virus (AAV) is detected by Toll-like receptor 9 (TLR9), a pattern recognition receptor found on the endosomal membranes of immune cells such as macrophages. TLR9 normally senses CpG motifs in the DNA genomes of viral and bacterial pathogens. When TLR9 senses AAV DNA, it similarly

results in innate immune and inflammatory responses. Prior studies showed that *Tlr9*-/- mice express higher and/or more durable levels of transgene expression compared to wild-type mice following AAV gene therapy. Thus, avoiding TLR9 activation may improve both safety and efficacy of gene therapy. One strategy for blocking TLR9 activation in cell culture is the administration of specific short single-stranded DNA oligonucleotides (typically shorter than 25nt) that bind TLR9 and antagonize its activation. We hypothesized that these TLR9-inhibitory sequences can retain functional activity when incorporated into a longer strand of DNA, thereby shielding the DNA from TLR9 activation. To test this, we selected a number of sequences that are known to antagonize TLR9 and fused them to an inflammatory CpG-containing oligodeoxynucleotide (ODN), ODN 2006 (24nt long). Using a HEK293-TLR9 reporter cell line, we found that this approach strongly reduced inflammation compared to ODN 2006 fused to random sequences, suggesting that TLR9-inhibitory sequences were able to function in cis. Next, we inserted three copies of ODN TTAGGG - a TLR9-inhibitory sequence derived from human telomeres - separated by short linkers, into the 3' untranslated region of a self-complementary AAV vector plasmid encoding eGFP ("AAV-eGFP-telomere"). When we infected primary human monocyte-derived macrophages with AAV2-eGFP or AAV2-eGFP-telomere, we observed that AAV2-eGFP triggered TNF secretion while the engineered vector significantly reduced TNF secretion. Intravenous or intramuscular administration to mice showed that AAV2-eGFP induced inflammatory and innate immune gene expression in the liver and muscle respectively 2h after treatment, while AAV2-eGFP-telomere was able to evade stimulating robust immune responses. Furthermore, we found that AAV2-eGFP-telomere resulted in multi-fold higher eGFP gene expression in both liver and muscle compared to AAV2-eGFP, 2-4w after AAV administration. We engineered an AAV8 vector expressing human factor IX (hFIX) under the control of the liver-specific transthyretin promoter using the same principles, and observed a ~2-fold increase in plasma hFIX protein levels compared to the parental vector. Our work demonstrates that incorporating short TLR9-inhibitory sequences within a longer piece of DNA, such as an AAV genome, can "cloak" the genome, preventing innate immune and inflammatory responses and improving the efficacy of the therapy. This approach may guide the design of future nucleic acid-based therapeutics.

1002. Therapeutic Factor IX (FIX) Activity after Single Treatment with AMT-060 (AAV5-FIX) in Hemophilia B Patients with Pre-Existing Anti-AAV5 Humoral Immunity

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Adeno-associated virus (AAV) neutralizing antibodies (NAB) are believed to diminish the efficacy of AAV-based therapies delivered systemically in humans. Anti-AAV NAB recognizing serotypes 2 and 8 are reported to interfere with initial transgene expression in preclinical and clinical studies. Levels of pre-existing anti-AAV NAB in humans at titers as low as 1:17 for AAV2 capsid (Manno et al, 2006) and even 1:1 for bioengineered capsid (AAV-Spark100; George et al., NEJM 2017) were associated with a total lack of or reduced therapeutic efficacy. Those observations have led to the exclusion of subjects with even low levels of anti-AAV NAB from current AAV-based gene therapy trials. We explored whether anti-AAV5 NAB also reduced the efficacy of gene therapy delivered by an AAV5 capsid. **Methods** Ten Hemophilia B patients entered our gene therapy clinical trial with AAV5-FIX (AMT-060). At screening for eligibility, no patient tested positive for anti-AAV5 NAB using a GFP-based bio-assay for detection of anti-AAV5 NAB and therefore, all were included in the study. We recently re-analyzed the pre-treatment sera of these patients using a more sensitive luciferase-based assay to detect anti-AAV5 NAB. The results obtained were correlated with FIX activity after treatment with AMT-060, AAV5-specific T-cell mediated immunity determined by human interferon gamma Elispot assay, and transaminase levels. Furthermore, pre-treatment sera of non-human primates (NHP) administered intravenously with AMT-060 were analyzed with the same luciferase-based anti-AAV5 NAB bio-assay used for the human study sample re-analysis. The relationship of pre-existing anti-AAV5 NAB titers with the liver transduction efficacy of AMT-060 was evaluated. Transduction efficacy was assessed by measuring the human FIX transgene proteins levels in NHP plasma as well as the vector DNA levels in the liver tissue. **Results and Conclusion** Using the luciferase-based anti-AAV5 NAB assay, 7/10 patients returned results below the limit of detection and 3/10 had positive titers. No relationship was detected between the presence of pre-treatment anti-AAV5 NAB and therapeutic efficacy: The patient with the highest anti-AAV5 NAB titer (340) presented the highest mean FIX activity (6.8%) in his dose cohort. The other two positive patients had titers of 210 and 21, with a mean FIX activity of <2% and 3.0%, respectively. None of the patients with pre-existing anti-AAV5 NAB experienced elevations in transaminases after-treatment. Additionally, no clinically relevant T-cell responses to the capsid were detected in any patient. The NHP sera were analyzed with the same luciferase-based anti-AAV5 NAB assay used for the human study. All animals showed pre-existing anti-AAV5 NAB, with titers ranging from 56 to 1030. Remarkably, effective and comparable AAV5 transduction efficacy was achieved in all animals, independently of the level of pre-existing anti-AAV5 NAB detected. In summary, successful liver transduction was achieved with AAV5 vector in both NHP and human at levels of pre-existing anti-AAV5 NAB titer up to 1030 for NHP and 340 for humans. These results demonstrate efficacious systemic gene delivery with AAV5 vector targeting the liver in presence of pre-existing anti-AAV5 NAB.

1003. Intradermal Delivery of a Synthetic, Consensus DNA Vaccine against Zika Virus, Gls-5700, is Highly Immunogenic in Humans Inducing Humoral and Cellular Immunity That is Maintained for over One Year

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Zika virus (ZIKV) is usually a self-limited infection associated with mild to no symptoms, as well as more rarely, Guillain-Barre syndrome. However, infection of women during pregnancy can lead to severe congenital birth defects. There are no approved therapies or vaccines for ZIKV. We have previously described that a synthetic DNA vaccine delivered by CELLECTRA electroporation leads to robust immune responses in mice and non-human primates (NHPs) and that these responses are protective in a mouse challenge model. We recently published a preliminary report detailing the early results from Zika-001, an open label, Phase I clinical trial (NCT02809443), that evaluated the safety and immunogenicity of GLS-5700, a synthetic, consensus DNA vaccine targeting the ZIKV pre-membrane/membrane and envelope proteins (prME). Here we report the long-term immunogenicity results from this trial. Vaccine doses of 1 or 2 mg were delivered intradermally followed by electroporation with the CELLECTRA-3P device at weeks 0, 4, and 12 to two groups of 20 participants. Cellular and humoral immune responses were assessed throughout the trial and out to week 60. Immunization with GLS-5700 resulted in robust humoral and cellular immune responses. After the third immunization, 100% of participants had an increase in binding antibody titers compared to baseline. More than one year later, 84% of participants in the 2 mg group maintained detectable binding antibodies with an average endpoint titer at week 60 of nearly 10^4. Passive transfer experiments previously demonstrated that post-immunization human serum was able to protect mice from lethal infection independent of neutralization titer. Antigen-specific cellular immune responses detected by IFNγ ELISPOT kept increasing in the 2 mg group through week 36 and were maintained for more than a year. An overall response rate of 89% in the 2 mg dose group was observed at both week 36 and week 60, more than 1 year after the first immunization. The Zika-001 Phase 1 study demonstrated the safety and immunogenicity of the GLS-5700 DNA vaccine for Zika virus in humans. The robust humoral and cellular responses in addition to their long-term maintenance in trial participants makes this a very promising Zika vaccine candidate deserving of further study in clinical trials.