AAV Vector Biology

1. Wild-Type and Recombinant AAV Integration in Human Cardiomyocytes: Focus on Mitochondrial Genome

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Recombinant adeno-associated viruses (rAAV) have been shown to integrate within the mitochondrial genome (mtDNA) in human and mouse skeletal muscle upon AAV-1 intramuscular delivery. However, mitochondrial AAV integration has not been further explored and the relevance of this finding with regards to AAV potential for the treatment of mitochondrial diseases is still unknown. Here we studied AAV mitochondrial integration in human cardiomyocytes by comparing the integration profile of both wild-type (wt) and rAAV. To this aim, human immortalized cardiomyocytes were infected with wild-type and rAAV-2 at an MOI of 50,000 viral particles per cell. AAV integration sites (IS) were amplified by LAM-PCR and subsequent data mining allowed identifying and mapping vector-genome junctions. We analyzed 1,254,997 LAM-PCR-derived sequencing reads and identified 14,048 and 2,396 IS from wtAAV- and rAAV-infected cells, respectively. Next, we performed common integration site (CIS) analysis on the IS retrieved in order to identify those genomic regions multiply targeted by wtAAV and rAAV. A total of 1,537 CIS were retrieved from wtAAV-infected cells, where only 12 CIS presented an order higher than 4. AAVS1 locus (PPP1R12C) figured among the high order CIS (order 11) and we also identified two high order CIS (order 33) within the OR4F29 and PCBD2 genes. As OR4F29 and PCBD2 are located within nuclear mitochondrial DNA segments (NUMTs), we performed a detailed analysis of the IS included within these two CIS. Within OR4F29 and PCBD2, we found that 57/69 IS exhibited a higher homology with mtDNA, 2/69 rather aligned to the nuclear genome and 10/69 were not distinguishable as they exhibited the same nuclear and mtDNA homology. On the other hand, rAAV-infected cells presented a more heterogeneous IS distribution and CIS analysis yielded a lower number of CIS, 100 in total, where only 2 had an order higher than 4. In this case, we focused on the 12 CIS presenting an order higher than 3. As expected, no IS within the AAVS1 locus. Although with lower orders, CIS within the OR4F29 (order 7) and PCBD2 (order 3) genes were also retrieved for rAAV. In this case, all the IS retrieved within these genes (10/10) exhibited higher homology with mtDNA. Our data show that both the wild-type and the recombinant AAV-2 integrate within the mitochondrial genome and, together with previous studies, this may indicate that rAAV can target the mitochondrial genome in cells exhibiting a high mitochondrial content. Thus, rAAV might constitute a promising tool for the treatment of mitochondrial disorders in tissues such as the skeletal muscle or the heart.

2. Novel AAV Vector Reservoirs: Peripheral Blood Cells and Hematopoietic Progenitors

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Upon systemic administration, recombinant adeno-associated viral vectors (rAAV) are cleared out from serum within 4-8 weeks. However, a sustained vector persistence was detected in peripheral blood mononuclear cells (PBMC) from patients receiving a rAAV-2/5 aimed to treat acute intermittent porphyria. Integration site (IS) analysis performed on these samples revealed the presence of genome-wide distributed integrated vector sequences with no vector integration hotspots nor IS clonal expansion.

rAAV persistence in PBMC was then further studied in the non-human primate (NHP) model. Quantitative (q) and linear amplification-mediated (LAM)-PCR were performed on PBMC collected 6 and 12 months following intravenous rAAV-1 or rAAV-2/5 administration. Similarly, bone marrow mononuclear cells (BMNC) collected 12 months after vector injection were analyzed to investigate whether PBMC vector persistence could arise from bone marrow transduction.

rAAV genomes persisted within PBMCs at all time points analyzed and LAM-PCR allowed to identify 187 IS. Interestingly, most of the rAAV sequences retrieved corresponded to concatemeric vector forms and, in line with a mainly episomal vector persistence, we detected a 1.5-fold decrease in vector copy number (VCN) and a reduction in the percentage of concatemeric forms (88.6 and 75.1% at 6 and 12 months, respectively). In BMNC, we found a 1-2-fold higher VCN when compared to PBMC that also correlated with a higher IS retrieval, 342 IS were identified. The vector was again found to persist mainly as concatemeric forms (93.8%). In order to determine whether rAAV was persisting homogenously within the progenitor and non-progenitor fractions of the hematopoietic compartment, $CD34^+$ and $CD34^-$ cells were isolated from BMNC. Importantly, higher VCN were retrieved within the hematopoietic progenitor compartment with an average 3.7-fold increase. Remarkably, IS retrieved from both PBMC and BMNC were genome-wide distributed with no targeting of cancer-related genes.

Therefore, we show that rAAV-1 and rAAV-5 are able to persist within NHP hematopoietic progenitors with neither clonal outgrowth nor signs for malignant transformation. This indicates the safe integration profile of these vectors within this compartment and points to rAAV as potential candidates for bone marrow gene therapy.

3. Role of Truncated Recombinant AAV Genomes in Tumor Formation

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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. Albeit 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 a mouse model. Male C3H/HeJ mice, in which liver tumors grow rapidly, were used to achieve the sensitivity needed to detect rAAV-associated oncogenic events. Genomic DNA from tumors of mice treated with a GFP vector driven by a cytomegalovirus (CMV) promoter was extracted, fragmented, and enriched for vector sequence using biotinylated RNA probes. The enriched DNA was sequenced on the HiSeq platform and analyzed by NCH Genomics Core staff for vector genome junctions, defined as any split sequence read that aligned to both the mouse and vector genomes. 5 of 8 tumors tested had integration sites within the second intron of the gene encoding the E3 ubiquitin ligase, ITCH, a suspected oncogene. All five integrations contained only the CMV promoter element and the SV40 intron splice donor site, lacking the splice-acceptor and GFP coding region, suggesting that the vector was either truncated during or prior to integration. Analysis of episomal vector sequences from normal adjacent liver tissue showed that 14% of vector genomes were less than full-length, and 2% of the vector sequence contained only the CMV promoter and the SV40 intron splice donor site, similar to the integrated structures. The juxtaposition of the truncated vector intron within the second intron of ITCH, upstream of the ATG start codon, is predicted to give rise to a dysregulated fusion transcript, leading to ITCH overexpression and contributing to tumor formation. Given the size of ITCH intron 2 (25 kb), the dose of vector (2.5E10/kg), and an estimated integration frequency of 0.1-0.5%,each treated mouse is predicted to have approximately 10 vector integrations within that region. However, only truncated vector genomes were associated with tumors, suggesting that they pose a specific risk for genotoxicity. The number of ITCH-associated tumors recovered from this group of mice is consistent with the 2% of the vector genome population that had the equivalent truncation, suggesting the possibility that they were present in the vector stock prior to administration.

4. Assaying Patterns of rAAV Integration in Humanized Mice: Implications for rAAV Genotoxicity

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Numerous gene therapy clinical trials that utilized recombinant adeno-associated virus (rAAV) as a vector are planned, underway or have been completed. To date these clinical trials have not reported severe adverse events related to rAAV leading to the belief that this vector is well-tolerated, and safe. However, numerous studies have reported hepatocellular carcinoma (HCC) formation after rAAV treatment of mice with a variety of disorders, including MPS type VII, phenylketonuria, OTC deficiency, molybdenum cofactor deficiency, hemophilia B, Sandoff disease, and methylmalonic acidemia (MMA), and a few of these studies have demonstrated a correlation between rAAV integrations and genotoxicity. In addition, a recent claim that insertional mutagenesis by wild-type AAV serotype 2 may contribute to the development of HCC in a small number of patients. The developing controversy surrounding possible AAV genotoxicity highlights the need for comprehensive studies to determine rAAV integration profiles in human hepatocytes. Although post treatment liver biopsy specimens would represent the ideal samples to use for the analysis of rAAV genotoxicity, we have developed an alternative approach, and studied hepatic rAAV integrations in humanized mice. Fah-/-, Rag-/- Il2rg-/- mice successfully repopulated with human hepatocytes were treated as young adults with $3X10^{11}$ GC of either AAV8 (n=1) or AAV9 (n=3) CBA GFP reporters and then the livers were harvested 10 days post injection. We used a previously described high-throughput integration site-capture technique with subsequent high-throughput sequencing to identify rAAV integrations, mapped the reads to either the mouse or human genome, and further characterized. In the 4 livers studied, there were a total of 2762 unique rAAV integrations. In the mouse genome; 1346 were located within genes, with 14 genes were shared across samples. 1148 unique rAAV integrations mapped to the human genome; 612 were located within genes and 3 genes contained integrations across samples. Furthermore, 69 genes were common between mouse and human genomes. We did not document rAAV integrations into Rian or TERT but the fact that a number of loci appeared recurrently, and between species, suggests that humanized mice will be useful to discover common rAAV integration sites, and explore potential genotoxicity associated with gene therapy.

5. The Multi-Serotype Receptor AAVR Interacts with AAV2 and AAV5 via Separate Domains

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Adeno-associated virus (AAV) entry is determined by its interactions with specific glycan receptors and its proteinaceous receptor(s). AAVR (also named KIAA0319L) is an important cellular receptor that is essential for the transduction of vectors derived from multiple distinct AAV serotypes including AAV2 and AAV5. Here, we further biochemically characterize the AAV-AAVR interaction and define the domains within the ectodomain of AAVR that bind to AAV. Using a virus overlay assay it was previously shown that the major AAV2 binding activity in membrane extracts corresponds to a glycoprotein with a 150-kDa molecular mass. By establishing a purification procedure, further separation through two-dimensional electrophoresis and mass spectrometry we now show that this protein is identical to AAVR. We show that AAVR is N-linked glycosylated but that this glycosylation is not required for AAV2 binding nor functional transduction. Using a combination of genetic complementation with deletion constructs and viral overlay assay with individual domains, we establish that AAV2 functionally interacts predominantly with the second most distal PKD repeat domain (PKD2) present in the ectodomain of AAVR. Interestingly, AAV5 is completely independent from this domain and instead requires the most distal PKD domain (PKD1). These results suggest that despite their shared dependence on AAVR as essential receptor, different AAV serotypes have evolved separate interaction interfaces to engage the same receptor. SP and WZ contributed equally to this work.

6. A Single Amino Acid in AAV Capsids Regulate the Requirement of the Assembly Activating Protein (AAP) for Assembly

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The adeno-associated virus (AAV) assembly-activating protein (AAP) is a non-structural viral protein coded within the cap gene that has recently emerged as an important regulator of capsid assembly. Each AAV serotype has its own cognate AAP; however, our cross-complementation assay of the common serotypes, AAV1 to AAV12, showed that non-cognate interactions between AAPs and capsid VP proteins (VPs) can also support capsid assembly of all the serotypes efficiently except for AAV4, 5 and 11, which we have found do not require AAP for assembly. Intriguingly, and opposed to our expectation, AAV12, the closest phylogenetic neighbor of AAV11 among the 12 serotypes, did not show AAP independency in capsid assembly. This observation prompted us to identify the amino acid(s) in the AAP-independent AAV11 VP and AAP-dependent AAV12 VP proteins responsible for the distinct difference in capsid assembly between these two serotypes. Here we show that a single amino acid at the same topological location determines whether assembly requires AAP (position 721 in AAV11 VP1 and 730 in AAV12 VP1, which are at the same alignment position because the AAV12 VP1 protein is longer than the AAV11 VP1 protein by 9 amino acids). To understand the mechanisms conferring AAP-independency, we first performed a C- and N-terminal domain swapping experiment between AAV11 and AAV12 VPs and found that the responsible region should reside within the C-terminal half of the VPs. Second, we bioinformatically identified 7 amino acids near the C-terminus that differ between the two serotypes and are potentially responsible for the AAP independency. Based on these amino acid differences, we created the following four AAV12 VP mutants, each carrying 2 to 7 amino acid substitutions to the corresponding amino acids in the AAV11 VP protein: AAV12-VI, -AVIKTPY, -VKTPY and -TPY (note: amino acid letters are those from AAV11 VP). These chimeric VPs were investigated for AAP independency in their capsid assembly by our cross-complementation assay. This analysis revealed that the TPY in the AAV11 VP confers AAP-independency on AAV12. To further dissect the responsible amino acid(s) within the TPY, we systematically introduced reciprocal mutations between the AAV11 and AAV12 VPs in all possible combinations and investigated gain of function of AAV12 mutant capsids (i.e., whether AAV12 capsid assembly becomes AAP-independent) as well as loss of function of mutant AAV11 capsids (i.e., whether AAV11 capsid assembly becomes AAP-dependent). This analysis revealed that a single L-to-P mutation at AAV12 position 730 and a single P-to-L mutation at AAV11 position 721 could invert the AAP-associated phenotypes in the capsid assembly. Thus, our study sheds light on the mechanisms of capsid assembly, highlights the importance of the 2-fold axis for assembly, and provides valuable insights about the differential role of AAP in the virus life cycles across different AAV serotypes.

7. Evolutionary Interrogation of AAP-Dependency on AAV Assembly Highlights Mechanism and Structural Determinants

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Adeno-Associated Virus (AAV) has earned increasing clinical validation as a therapeutic gene delivery vector. To meet the need for more refined delivery for specific disease targets, capsid engineering to improve transduction or immunological properties is actively pursued. To support these efforts, we aimed to provide a deeper understanding of structure-function relationships of the AAV virion, with a central focus on mechanisms of capsid assembly—a primary requirement for any vector. The Assembly Activating Protein (AAP) was previously demonstrated to be an essential viral co-factor in capsid assembly, but to date studies are limited to a VP3-only context. We tested the requirement of AAP for capsid assembly within the full complement of VP proteins and in the natural transcriptional context of the AAV genome by introducing early stop codons into AAP's reading frame of the *cap* gene for 12 naturally occurring serotypes and 9 functional intermediates of these 12 serotypes, which we previously generated by ancestral sequence reconstruction, and which comprise a putative AAV capsid phylogeny. Requirement of AAP for virion assembly exhibited a continuum from full independence (e.g. AAV5), to strict dependence (e.g. AAV8). Alternative placement of the early stop codon revealed that this continuum can be divided into three categories: capsids that require a full length AAP protein, capsids that require only the N-terminal portion of AAP (AAP-N), and capsids that do not require AAP for assembly.

To assess what drives the AAP phenotype at the molecular level, we interrogated levels of VP protein expressed from these constructs. AAP-independent serotypes maintain high VP levels in the absence of any AAP, and AAP-dependent serotypes require a full length AAP to achieve appreciable VP levels. AAP-N promotes high VP levels in serotypes with intermediate AAP phenotypes; low quantities of assembled virions in these serotypes suggest that the C-terminal portion of AAP (AAP-C) provides an assembly function distinct from AAP-N's stabilizing role.

We next aimed to identify structural elements of VP that contribute to AAP-independent assembly, and shed light on AAP-C's function. Phylogenetic nodes illustrate divergence in AAP phenotype across a small genetic distance, which we explored to fine-map structural motifs required for self-assembly versus AAP-assisted assembly. Partial AAP-independence is transferrable to a strictly AAP-dependent serotype by engraftment of the identified motif onto a heterologous capsid. This mutant gains VP stability functionality, losing the requirement for a full-length AAP. The majority of the sites in this motif lie at the VP trimer interface, suggesting that self-oligomerization contributes to VP protein stability and AAP-independent assembly, and that AAP-C functions to promote VP oligomerization. Our studies lend new insight into capsid assembly mechanisms and suggest a model with a dual role for AAP in early AAV assembly steps.

8. Vector Dose-Dependent Delayed CD8+ T Cell-Mediated Clearance of AAV Encoded Antigen in Liver

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Tolerogenic properties of the liver make it an attractive target organ for gene therapy of inherited protein deficiencies, such as hemophilia, metabolic disorders, lysosomal storage disorders, etc. At the same time, these features may keep the immune system from effectively eliminating hepatotropic antigens such as observed in malaria, hepatitis B and hepatitis C viruses, leading to chronic infection. Previously, we demonstrated that the generation of a systemic immune response to an AAV encoded antigen upon hepatic gene transfer is dependent on vector dose. Using 3 different doses (low: $1x10^8$ vg, medium: $1x10^9$ vg, and high: $1x10^{10}$ vg) of an AAV8 vector expressing ovalbumin (AAV8-OVA) in immune competent C57BL/6J mice, we showed dose dependent systemic expression of OVA, resulting in distinctly different immune responses at different vector doses. Although circulating OVA-specific $CD8^+$ T cells were not detected at the low and high doses, a high frequency (5-35%) of these cells were detected in 40-50% of mice at the medium dose. In these mice, despite circulating OVA-specific $CD8^+$ T cells detected within one month of gene transfer, systemic OVA expression lasted for more than two months. Though OVA-specific $CD8^+$ T cells were found to be highly efficient in *in vivo* cytolytic assay, delay in elimination of AAV8 transduced cells was attributed to expression of inhibitory molecules such as PD-1 and 2B4 on these cells. Further, loss of OVA expression at ~2.5 months correlated with down regulation of PD-1 expression. Therefore, an intact PD-1/PD-1L pathway may be required for the delay in elimination of OVA expressing liver cells. To test this hypothesis, we injected 6 PD-$1^{-/-}$ mice with medium dose ($1x10^9$ vg) of AAV8-OVA. Out of 6 PD-$1^{-/-}$ mice injected with AAV8-OVA, 3 (50%) had circulating OVA-specific $CD8^+$ T cells, similar to WT animals. Average frequency of OVA-specific $CD8^+$ T cells at 2 weeks PI was ~2.3% (range 0.15% - 5.95%), which increased to ~10% (range 2.6% - 16.3%) at 4 weeks PI. In contrast to wild-type mice, none of the PD-$1^{-/-}$ mice had systemic OVA expression at any of the time points tested (2 and 4 weeks), indicating rapid elimination of the target antigen when the PD-1/PD-1L pathway is disrupted. In addition, livers of PD$1^{-/-}$ mice had substantial $CD8^+$ T cell infiltrates. Significantly increased numbers of $CD8^+$ T cells were observed in liver sections of wild-type mice that lost expression despite absence of circulating OVA-specific $CD8^+$ T cells, suggesting that a localized immune response was responsible for the loss of expression in these animals. Overall, our results support that antigen load and/or distribution in the liver microenvironment (as a function of vector dose) plays a significant role and that immune checkpoint regulators (such as PD-1/PD-1L pathway) control $CD8^+$ T cell mediated immunopathology.

Oncolytic Viruses for the Treatment of Cancer

9. Oncolytic Measles Viruses as Therapeutic Vectors for Tumor-Directed BiTE Expression

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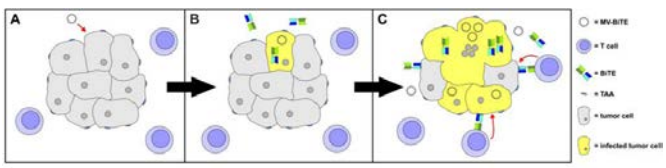
We generated oncolytic measles viruses (MV) encoding bispecific T cell engagers (BiTEs) to enhance anti-tumor immune responses in oncolytic virotherapy. Induction of anti-tumor immunity has emerged as the major aim in developing successful virotherapeutics and can be improved by the ability of certain oncolytic viruses to carry therapeutic transgenes. BiTEs are artificial antibodies comprising two single-chain variable fragments able to cross-link CD3 on T cells with tumor surface antigens. Thereby resting, polyclonal T cells can be recruited to lyse tumor cells, irrespective of T cell receptor specificity and antigen presentation. We hypothesize that tumor-restricted expression of MV-encoded BiTEs enhances local anti-tumor immune responses while minimizing systemic side effects. We engineered oncolytic measles virus constructs (MV-BiTE) to encode BiTEs targeting either human CD20 or CEA as tumor-associated antigens along with human and murine CD3, respectively.

Expression, binding and cytotoxic potential of MV-encoded BiTEs were validated in vitro. Time-course experiments demonstrated BiTE expression and secretion by cell lines infected with MV-BiTE. As determined by growth curves and cell viability assays, viral replication kinetics and oncolytic efficacy are not impaired compared to the unmodified virus. Specific binding to the relevant target antigens was verified by sandwich and competitive ELISAs, pull-down assays and flow cytometry. In co-cultures with freshly isolated PBMCs or murine T cells, BiTEs mediate specific tumor cell lysis and promote secretion of TH1 effector cytokines. Therapeutic efficacy of MV-BiTE was demonstrated in a novel, syngeneic tumor model of B16 cells stably expressing human CD20 as a BiTE target antigen and human CD46 as a measles virus entry receptor. Compared to the virus encoding non-targeting anti-CEA BiTE, treatment with MV-BiTE targeting CD20 significantly delayed tumor progression and prolonged survival.

Currently, we are performing efficacy studies in a unique humanized model of colorectal carcinoma with CEA-expressing human primary tumor spheres and human immune effector cells in NSG mice. Furthermore, we will investigate the mechanisms of MV-BiTE action by analyses of immune cell infiltrates and cytokine profiling. Conclusively, the use of MV as a vector for targeted expression of BiTEs represents a promising novel treatment strategy to support anti-tumor immunity while preventing side effects. Our results indicate potential for future clinical translation.



Model of MV-BiTE immunovirotherapy. (A) A BiTE-armed oncolytic measles virus (MV-BiTE) enters a tumor cell. **(B)** Upon infection, viral replication is initiated along with BiTE expression and secretion. **(C)** Infected tumor cells form syncytia with adjacent cells. Viral progeny are released and infect further neighboring tumor cells. Secreted BiTEs bind to their respective tumor surface antigen and to T cell receptor-associated CD3, resulting in tumor cell lysis by crosslinked T cells.

10. Immune System as a Determinant for Response to Oncolytic Measles Virotherapy

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While there has been documentation of successful responses to oncolytic virotherapy, outcomes can vary significantly among patients. In this study we set out to characterize potential mechanisms of resistance to Edmonston vaccine derived oncolytic Measles virus (MV) strains. We identified several primary glioblastoma (GBM) patient derived xenograft (PDX) lines that were permissive (GBM12, GBM43 and GBM64) or resistant (GBM39, GBM6 and GBM150) to MV replication and cell killing. Using GBM12 and GBM39 as a model of permissiveness and resistance, we demonstrated that productive virus replication *in vitro* corresponded to a therapeutic benefit *in vivo.* MV therapy resulted in significant prolongation in median survival (37 days (185% increase); p-value < 0.001) in mice orthotopically implanted with permissive GBM12 cells. In comparison, MV therapy in mice implanted with GBM39 cells demonstrated no benefit. To measure potential differences in virus entry, GBM12 and GBM39 cells were infected with MV-GFP (MOI 1.0) and GFP positive cells measured at 24 h post-infection. GBM12 and GBM39 were infected at comparable levels (29% and 24%, p-value n.s.), suggesting a mechanism of post-entry restriction. To identify potential mechanisms, we performed Next-Generation RNA-Seq analysis using RNA isolated from uninfected cells. Our gene expression analysis identified a pre-existing antiviral state in uninfected GBM39 cells, characterized by the constitutive expression of several interferon-stimulated genes (ISGs). In contrast, ISG expression in GBM 12 was at low or non-detectable levels. ISGs are the effector proteins in the interferon (IFN) pathway, which is part of the mammalian innate immune system that protects the host from potential pathogens. Upon virus detection, IFN-β is secreted and signals through the JAK/STAT pathway to induce an antiviral state in neighboring cells. Therefore, we hypothesized that the pre-existing antiviral state primes resistant cells to mount a rapid and robust antiviral response upon viral infection. To test this hypothesis we characterized the antiviral response to MV in GBM12 and GBM39 cells. IFN-β secretion was detected by 12 h post-infection (1,128 pg/ml) in GBM39 cells and peaked at 24 h (6,600 pg/ml). In contrast, a low level of IFN-β, 92.6 pg/ml, could only be detected at 24 h post-infection in GBM12 cells. The rapid induction of IFN-β coincided with STAT1 and STAT3 activation in GBM39 cells 4 h post-infection; whereas activation was delayed in GBM12 cells. In an effort to determine the role baseline ISG expression plays in MV restriction, cells were treated with a JAK inhibitor (Ruxolitinib). 48 h pre-treatment with Ruxolitinib (3 μm) significantly reduced the baseline expression of several ISG, as well as induction upon infection, as measured by quantitative RT-PCR. In order to differentiate the effects of the baseline level of ISG expression from the induced antiviral response, Ruxolitinib was added at different times during the course of infection. Interestingly, virus replication was increased >100-fold (p-value <0.05) when Ruxolitinib was added at the time of infection, 6 h and 12 h post-infection. However, removal of Ruxolitinib immediately prior to infection was insufficient to rescue virus production. Overall, these results suggest that the baseline antiviral state in resistant cells can enhance the antiviral response upon

infection. Furthermore, our results highlight important interactions that could be used to guide patient selection, oncolytic virus design and combinatorial strategies. Funding by: Brain SPORE (P50 CA108961), R01 CA154348

11. Type 1 IFN Response Is a Major Determinant of Oncolytic Measles Virus Activity in a Model of Sequential Stromal Cell 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. Although a few studies have provided vital mechanistic insights, studies directly comparing responses to MV infection in cancer cells and healthy normal counterparts are lacking. 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 following retroviral transfer of human telomerase reverse transcriptase (hTERT), human papilloma virus16 E6 and E7 (3H), SV40 small T antigen (4+V), finally, H-RAS (5H) (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 any differences in MV receptor 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. In order to identify genes associated with resistance to MV infection, as well as assess the genetic effects of MV infection in susceptible cell lines, whole genome expression profiling was performed using next-generation sequencing. 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. Furthermore, GEP also showed up-regulation of a subset of ISGs, among which ISG15, IFI6, IFITM1 and BST-2 are top hits, in response to MV infection, with hTERT cells showing larger fold increases compared to minimal up-regulation in 5H cells. To confirm the biological relevance of IFN production in MV-permissiveness, 5H cells were exogenously treated with IFNα/β. However, this did not seem to render the cells resistant to MV infection and compensate for their native type 1 IFN production. Altogether, our data suggests that basal innate immune responses are critical determinants of oncolytic measles virotherapy. Further analyses and validations of GEP data are ongoing to classify ISGs and other genes involved in specific pathways that may be correlated to MV resistance.

12. Combinatorial Treatment of "Armed" Oncolytic Adenovirus Expressing Checkpoint Inhibitor and Cytokine with Chimeric Antigen Receptor T-Cells Leads to Superior Anti-Tumor Effects in Head and Neck Cancer

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Oncolytic Adenoviral vectors (Onc.Ads) encoding an immunomodulatory transgene ("Armed"Onc.Ads) have promise as cancer immunotherapy agents. Intratumoral administration of "Armed" Onc.Ads cannot cure bulky and metastasized tumors, suggesting that additional immunomodulation is required to enhance host immune responses. Due to the limited transgene capacity of Onc.Ads, we combined Onc.Ad with Helper-dependent Ad (HDAd) (CAdVEC) to provide oncolysis in combination with the assisted replication of an HDAd that can express multiple immunomodulatory genes. Local CAdVEC expressing GM-CSF and IL-12p70 treatment was still insufficient to cure bulky and metastasized tumors. In contrast to oncolytic viruses, chimeric antigen receptor-modified T-cells (CAR T-cell) can actively traffic to primary and metastasized tumors. But while CAR T-cells have shown significant efficacy in hematological malignancies, their success in solid tumors has been limited. This is in part due to the inhibitory tumor microenvironment including expression of PD-L1. CAdVEC has a large transgene capacity, and we hypothesized that CAdVEC can provide both PD-L1 blockade and an activating cytokine to augment the activity of tumor directed CAR T-cells in the disrupted tumor environment produced by Onc.Ad. To address our hypothesis, we evaluated the anti-tumor effects of combinatorial treatment of CAdVEC expressing both a cytokine and PD-L1 mini-body with HER2.CAR T-cells in head and neck squamous cell carcinoma (HNSCC) models. HNSCCs are commonly a locoregional disease amenable to intratumoral virotherapy. Additionally, aberrant expression of HER2 is an indicator of poor prognosis in HNSCC patients. Using two HNSCC xenograft models, we screened HDAds expressing a different cytokine (IL-2, IL-7, IL-12p70, IL-15 or IL-21) combined with HDAd expressing PD-L1 minibody (HDAd*PD-L1*). Although infection of tumor cells with HDAds led to secretion of the cytokines they encoded, only the combination of HDAd*IL12p70* with HDAd*PD-L1* increased the anti-tumor effects of adoptively transferred HER2.CAR T-cells in vivo. We thus constructed an HDAd encoding both IL-12p70 and PD-L1 minibody expression cassettes (HDAd*IL12_PDL1*) to be co-administered with Onc.Ad, generating CAdVEC*IL12_PDL1*. We found that intratumoral CAdVEC*IL12_PDL1* treatment enhanced HER2. CAR T-cell persistence and expansion at the tumor site, prolonging animal survival more than 5-fold (> 100 days) in both xenograft

models compared to animals treated with only Onc.Ad or HER2. CAR T-cells alone (< 25 days). We also found that local treatment of CAdVEC*IL12_PDL1* with systemic treatment of HER2.CAR T-cells has significant anti-tumor effects to both primary and metastasized tumors in an orthotopic HNSCC model that produces lymph node metastasis, mimicking the pattern seen in HNSCC patients. Our data indicate that the CAdVEC platform, in conjunction with CAR T-cell therapy, can provide multi-faceted immunomodulatory support to enhance anti-tumor activity.

13. Targeting Historically Recalcitrant Colorectal Cancer Cells: Novel Oncolytic Chimeric Poxvirus Is Imageable and More Potent Than Predecessors

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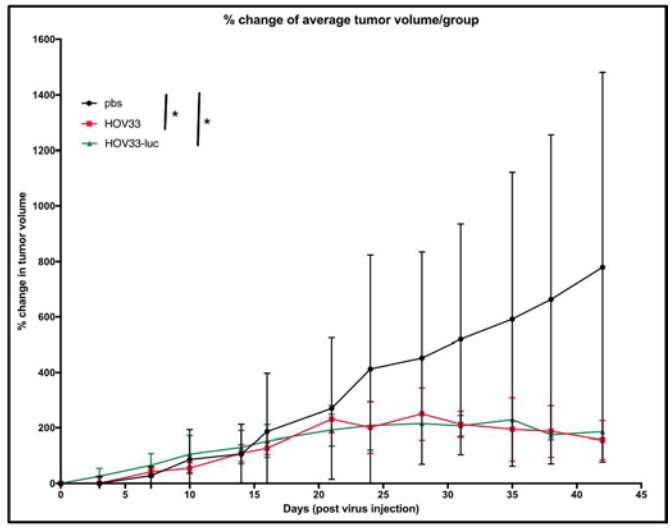
Introduction: Oncolytic viral therapy has shown preclinical and clinical promise, but the large amount of virus needed for adequate dosing results in extreme expense. More potent vectors are needed to create sustainable therapy regimens. This study demonstrates enhanced potency of a novel chimeric poxvirus capable of killing colorectal cancer cells known to be non-responsive to other oncolytic vaccinia vectors.

Methods: Using a novel method of chimerization and high throughput screening, a new super-potent chimeric orthopoxvirus (HOV-33) was constructed. The *J2R* thymidine kinase (*tk*) locus was replaced with either green fluorescent protein (GFP) or luciferase (*luc*) expression cassette (HOV33-gfp; HOV33-luc). *In vitro* cytotoxicity and viral replication studies at different multiplicities of infection (MOI) were performed on various colorectal cancer cell lines, including HT-29 which has previously proven non-responsive to other oncolytic vaccinia vectors. *In vivo* HT-29 flank xenografts were implanted in athymic mice, and when tumors reached approximate volume of 200mm^3, a single dose of either intratumoral or intraperitoneal virus was delivered. Biodistribution and viral replication were evaluated in real-time using bioluminescence imaging as well as virus titration in mouse organs harvested at a pre-determined time points. Tumor size was monitored at regular pre-determined intervals using digital caliper measurements. Mouse weights and behaviors were closely monitored.

Results: HOV-33 infects, replicates in, and kills HT-29 colon cancer cells in a dose-dependent manner. Viral replication and cytotoxicity do not appear to change with *tk* knockout. HT-29-derived flank xenografts treated with a single intratumoral or intraperitoneal injection (1 x 10^5 plaque forming unit) of HOV-33 or HOV33-luc stabilized tumor growth or showed tumor regression regardless of delivery route. HOV33-luc viral efficacy *in vivo* correlates with bioluminescence imaging, and tumor-specific viral replication is suggested by luciferase signal in tumors alone. Figure 1 shows HOV33 & HOV33-luc efficacy against HT-29 xenografts.

Conclusions: Novel oncolytic chimeric poxviruses are effective against colon cancer cell lines previously non-responsive to vaccinia infection. These vectors also induce tumor stabilization or regression at a dose 50 fold lower than doses employed by other investigators against responsive colon cancer xenografts, indicating enhanced viral potency and efficacy. Insertion of luciferase at the *tk* locus yields real-time imaging of viral replication and correlates with tumor response.



P < 0.05

14. Development of the Novel miRNA Engineered Oncolytic Virotherapy for Clinical Trial

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Oncolytic virotherapy has joined the ranks of cancer treatments in the past two decades and several viruses are considered to be used clinically in short. These therapeutic viruses selectively infect and lyse the tumor cells and induce systemic anti-tumor immunity. In a previous study, we have demonstrated that coxsackievirus B3 (CVB3) is a potent and novel oncolytic agent with direct lysis of human non-small cell lung cancer cells (NSCLC). And two organ-specific(muscle and pancreas) miRNAs target sequences were constructed into the 3' untranslated region of the CVB3 genome (CVB3-miR1&217T), markedly attenuated CVB3-induced pancreatitis and myocarditis. However, non-clinical acute toxicity testing of recombinant CVB3 in mice and monkeys showed that mild abnormalities of hematology and histopathology tests were observed in high-dose group.

To enhance its safety profile, we focused on two mircoRNAs-miR-34a and miR-34c, which are constitutively expressed in normal organs but down-regulated in various cancers. We have successfully genetically constructed two novel recombinant CVB3-miRTs (CVB3-miR34a and CVB3-miR34c) by inserting target sequences for miR-34a or miR-34c into 3' untranslated region of the CVB3 genome, respectively. *In vitro* crystal violet staining assays were performed to compare the cytotoxicities of both wild-type CVB3 and CVB3-miRTs infection

in various cancer cell lines. These results showed that infection with CVB3-miRTs in NSCLC and pancreatic cancer cells retained its abundant viral replication in comparison with the parental CVB3, while its attenuated replication was observed in human normal lung cell line that expressed high level of miR-34a and miR-34c, demonstrating the dependence of its infectivity on miR-34a or miR-34c levels. *In vivo* studies using mice to confirm the increased safety are going to be done.

Our results demonstrate that microRNA-targeting strategy to control viral tissue tropism and pathogenesis without attenuating oncolytic effect will be useful for the clinical translation of CVB3 virotherapy.

15. Immune Stimulation and Immune Checkpoint Nhibition Provided by a Single Agent: An Oncolytic HSV Expressing scFv Antibody Against PD-1

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Glioblastoma multiforme (GBM), a deadly brain tumor, is resistant to current standard of care treatments including surgery and chemo-radiotherapy. This cancer is also characterized by a highly immunosuppressive microenvironment. Immune checkpoints inhibitors (such as the antiPD-1 antibody) efficiently restore T-cell activity and have been recently approved by the FDA for the treatment of several cancers. Oncolytic viruses (OVs), self-replicating biologic agents for tumor, represent a new class of immunotherapy. OVs are thought to mediate antitumor activity through a dual mechanism: selective replication and lysis of infected cancer cells and induction of host antitumor immunity. I hypothesize that an oncolytic HSV (oHSV) can disrupt the immunosuppressive GBM microenvironment and stimulate anti-tumor T cell immunity. Further, combining this with PD-1 blockade locally in the tumor could maximize antitumor immune effects. Therefore, I developed a new oHSV that expresses a single-chain variable fragment (scFv) antibody against mouse PD-1 to block the PD-L1:PD-1 axis signaling pathway. Preliminary experiments demonstrate that this new oHSV1 (called oHSVscFvPD-1) expressed and secreted the scFv antibody in several mouse glioma cell lines retaining the same cytotoxic activity of the parental virus (oHSV). An *in vivo* orthotopic glioma mouse model resulted in a significant improvement of median survival time with oHSVscFvPD-1 compared to untreated mice (69 vs 22 days) and in a higher percentage of long-term survivors mice (surviving more than 120 days) when treated with oHSVscFvPD-1 compared to parental oHSV (43 vs 37%). Long-term survivors mice have been also re-challenged with the same mouse glioma cell line in the contralateral hemisphere showing a memory response against the tumor. Overall, the results obtained by my experiments could then justify testing the new oHSVscFvPD-1 virus in a phase I human clinical trial and/or lead to additional approach for the treatment of human glioblastoma.

16. Phase 2 Trial Evaluating Biodistribution and Shedding of Talimogene Laherparepvec (T-VEC) in Patients (Pts) with Unresectable Stages IIIB/IV Melanoma

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Background: T-VEC is a first-in-class, engineered herpes simplex virus (HSV) type-1, used to treat metastatic melanoma. To date, no direct transmission of T-VEC from pts to close contacts (CC) has been reported. This phase 2 study (ClinicalTrials.gov, NCT02014441) analyzed T-VEC biodistribution, shedding from injected tumors, and transmissibility risk to CC among pts with stage IIIB-IV melanoma after intralesional T-VEC treatment. **Methods:** T-VEC was injected into cutaneous, subcutaneous, and nodal melanoma lesions (first dose, ≤4 mL×10^6 pfu/mL; after 3 weeks, ≤4 mL×10^8 pfu/mL Q2W) for ≥6 months until complete response, absence of injectable lesions, clinically relevant disease progression, or intolerance. Injected lesions were to be swabbed with alcohol before/after injection and covered with occlusive dressings for ≥1 week. DNA samples were collected from blood, urine, injection sites, and dressing exterior during cycles 1-3 and 30 days after treatment end, from the orolabial and anogenital regions weekly during cycles 1-3, then after each subsequent cycle, and from days 30-60 after treatment end, and from herpetic lesions reported by pts/CC. T-VEC DNA was measured by qPCR; infectious virus in positive swabs was detected via 50% tissue culture infective dose ($TCID_{50}$) assay. **Results:** 60 pts received ≥1 T-VEC dose; 55% were men, median (range) age was 65 (19-93) yrs, and 75% had ECOG status 0. 17% were stage IIIB, 53% were stage IIIC, 30% were stage IV, and 67% were HSV-1 seropositive at baseline. Median (range) number of T-VEC injections was 10 (2-36). During treatment, T-VEC DNA was detected in the blood and urine of 59 (98%) and 19 (32%) of pts, respectively. Highest levels occurred during cycle 2; none occurred after treatment end. All pts had detectable T-VEC DNA on the surface of injected lesions at some time during treatment. 8 samples from 7 pts (12%) tested positive for infectivity per $TCID_{50}$ assay. All positive samples were from cycle 1 or 2. 48 pts (80%) had detectable T-VEC DNA on the dressing exterior; 7 pts (12%) had detectable T-VEC DNA in oral mucosa swabs. 5 samples from 4 pts (15%) had detectable DNA in anogenital swabs. 36 swabs (from 19 pts) of lesions of possible herpetic origin were taken; 4 swabs from 3 pts had detectable T-VEC DNA. 3 CC reported exposure or signs/symptoms of suspected herpetic origin; no T-VEC DNA was found in either of 2 available samples. Other than injected lesions, no other PCR-positive swabs tested positive by $TCID_{50}$; no swabs were PCR-positive after end of treatment. **Conclusion:** Biodistribution and shedding data suggest the highest risk of potential exposure to live T-VEC virus is from touching injected melanoma lesions. The risk of T-VEC transmission from pts to CC is low with proper handling/administration and post-injection care.

Genome Editing: Transcriptional Regulation and Specificity

17. Activity and Specificity of Engineered CRISPR-Cpf1 Nucleases in Human Cells

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CRISPR nucleases have been widely useful for a variety of genome editing applications to knockout genes, introduce specific changes, or to regulate gene expression, demonstrating their vast potential as basic research and therapeutic tools. Recently, CRISPR-Cas Cpf1 nucleases have been described(1) that bear a number distinctive features relative to the canonical Cas9 nuclease from *Streptococcus pyogenes* (SpCas9). Whereas SpCas9 recognizes a guanine-rich protospacer adjacent motif (PAM) of the form NGG on the 3' end of a target sequence, most Cpf1 orthologues described to date recognize a T-rich PAM on the 5' end of the target sequence(1). Furthermore, Cpf1 leaves a 5' overhang of 3 to 5 nucleotides in length instead of a blunt double-strand break, and also uses only a single short ~40 nt crRNA instead of the nearly 100 nt single guide RNA (sgRNA) commonly used with SpCas9. Because of these potentially advantageous features, we sought to characterize two Cpf1 orthologues from *Acidaminococcus sp. BV3L6* and *Lachnospiraceae bacterium ND2006* (AsCpf1 and LbCpf1, respectively) to examine their usefulness as genome editing reagents.

Robust on-target activity and genome-wide specificity are two desirable properties for engineered nucleases. Based on assessment of on-target activity across more than 40 target sites, we demonstrate that both AsCpf1 and LbCpf1 function robustly in human cells with efficiencies comparable to those of SpCas9(2). We also establish that most nucleotide positions across the spacer sequence targeted by AsCpf1 and LbCpf1 are highly sensitive to single or double base substitutions, suggesting that the enzymes possess high specificity. Consistent with these results, GUIDE-seq performed in multiple cell types followed by targeted deep sequencing analyses to examine genome-wide specificity reveal no detectable off-target cleavage for over half of 20 different crRNAs we examined. Combined, our results suggest that the two Cpf1 nucleases we characterized generally possess robust on-target activity and high specificities in human cells.

Because both AsCpf1 and LbCpf1 recognize extended T-rich PAMs of the form TTTN, Cpf1 targeting range is restricted relative to that of SpCas9 and other Cas9 orthologues. We therefore sought to use structural information available for AsCpf1 to improve targetability by relaxing or altering PAM specificity. We have identified a series of engineered PAM variants of AsCpf1 that more than double its targeting range and that have a minimal impact on mismatch tolerance. We will also describe how mutations analogous to those present in our previously described high-fidelity SpCas9 (SpCas9-HF) variants(3) can be implemented to retain high genome-wide specificity as needed for Cpf1 nucleases.

Overall, our results suggest that both natural and engineered forms of Cpf1 nucleases are robust and useful enzymes, findings that should encourage broader implementation of these genome editing reagents.

(1) Zetsche (2015) *Cell* (2) Kleinstiver and Tsai (2016) *Nature Biotechnology* (3) Kleinstiver and Pattanayak (2016) *Nature*

18. Targeted Multiplex Regulation of Endogenous Human Genes Using Drug-Inducible CRISPR-Cpf1-Based Transcription Factors

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The ability to regulate gene expression in a targeted and inducible fashion will enable numerous research applications and may provide the basis for novel therapeutics to treat human diseases. Previously published work has used engineered zinc fingers, arrays of transcription activator-like effector domains, or catalytically inactive "dead" CRISPR-Cas9 (**dCas9**) proteins to recruit various effectors or enzymes to a specific genomic locus of interest. Examples of domains recruited in this way include transcriptional activators or repressors, histone modifying enzymes, or enzymes that modify the methylation status of DNA.

Here we describe the development of a drug-inducible CRISPR-Cpf1-based system for targeted gene regulation in human cells. RNA-guided Cpf1 nucleases have protospacer adjacent motif (PAM) preferences that differ from well characterized Cas9 orthologues and thus can be used to target a different range of DNA sequences. We introduced mutations into Cpf1 to inactivate its nuclease activity, thereby generating "dead" Cpf1 proteins (**dCpf1**). We then designed and tested a drug-inducible scaffold fused to dCpf1 that allowed us to recruit multiple transcriptional activation domains to specific target sites in human genes. These studies revealed that our dCpf1 platform could be used to robustly activate the expression of endogenous human genes in a drug-dependent fashion by recruiting various transcriptional activation domains such as VP64, NF-KB p65, and a hybrid VPR multimeric domain to a promoter-proximal target site. By varying the structure of the scaffold fused to the dCpf1 protein or the concentration of dimerizer drug, we found that we could titrate levels of gene activation. We also observed that different activation domains may be optimal for increasing expression from different endogenous gene promoters.

Another important advantage of Cpf1 nucleases is the ability to easily express multiple crRNAs (which play a major role in guiding Cpf1 to target DNA sequences) in a single transcript with subsequent processing of the individual crRNAs by Cpf1 itself. Leveraging this simple multiplex capability, we observed that we could vary the degree of transcriptional activation by altering the number of crRNAs targeted to a given promoter.The dCpf1-based system we describe here combines the simplicity of RNA-guided targeting, inducible control with a small molecule, and the capability to easily modulate the number of sites targeted using multiplex guide RNA expression.

Beyond its utility for gene activation, we envision that our drug-inducible, multiplex dCpf1-based platform will enable targeted recruitment of other heterologous proteins or functional domains to any endogenous genomic locus of interest.

19. Improvement of CRISPR Activity and Specificity via Proximal Binding of Multiple CRISPR/Cas Systems (proxy-CRISPR)

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Bacterial class 2 CRISPR/Cas systems comprise diverse effector endonucleases with different targeting ranges, specificities, and enzymatic cleavage properties, but many of them are found inactive in mammalian cells and are precluded from genome editing applications. To explore CRISPR/Cas genome editing capabilities, we characterized a set of CRISPR/Cas systems for their nuclease activities in human cells and observed a wide range of variability in nuclease activity. CRISPR systems tested included type IIA *Streptococcus pyogenes* Cas9 (SpCas9), type IIA *Streptococcus pasteurianus* Cas9 (SpaCas9), type IIB *Francisella novicida* Cas9 (FnCas9), type IIC *Campylobacter jejuni* Cas9 (CjCas9), type IIC *Neisseria cinerea* Cas9 (NcCas9), type V *Acidaminococcus sp* Cpf1 (AsCpf1), and type V *Francisella novicida* Cpf1 (FnCpf1). The widely adopted SpCas9 was the most active, though it was severely inhibited on certain target sites, whereas CjCas9 and NcCas9 were the least active. We hypothesized that eukaryotic chromatin structure may be a causal factor in CRISPR nuclease inhibition and attempted to change local chromatin structures via proximal binding of orthogonal Cas9 proteins. This proximal co-targeting strategy (termed proxy-CRISPR) was shown to restore the nuclease activity from undetectable levels to over 30% indels as measured by CEL-I mismatch assays. Most importantly for potential therapeutic applications, we found the proxy-CRISPR strategy can facilitate precise gene editing of a single target site despite the presence of identical target sites within two different human hemoglobin genes. Our findings offer a novel strategy to modulate CRISPR activity to enable use of diverse CRISPR systems that might otherwise be deemed inactive in mammalian cells and provide insights to advance our development of more effective CRISPR endonucleases for gene editing.

20. High-Throuput Screening Selection of microRNAs Enhancing Cas9-Mediated Homologous Recombination

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The definitive treatment of genetic diseases through precise gene editing has been a long sought goal of gene therapy, unachieved at clinical level yet. The advent of biotechnological tools that use the bacterial CRISPR-Cas9 endonuclease and an engineered single-guide RNA (sgRNA) for the manipulation of mammalian genomes has dramatically changed the perspectives of achieving high frequency gene correction in both primary cultured cells and tissues. Despite the overall excitement, however, gene correction through the precise homologous recombination (HR) machinery is largely less efficient than the error prone non-homologous end joining (NHEJ) in mammals, in particular in adult post-mitotic tissues, such as heart and brain. For this reason, we developed an in vitro fluorescence-based assay for the genome-wide, high-throughput identification of RNA regulators and enhancers of gene editing mediated by HR. In U2OS cells, we transiently transfected a combination of three plasmids: the reporter pCDNA3eGFP(Y66S) containing a mutated EGFP sequence coding for a misfolded, non-fluorescent protein; the plasmid pX330-U6-sgEGFP5-Chimeric_BB-CBh-hSpCas9 coding for Streptococcus pyogenes Cas9 and for the specific sgRNA cutting inside EGFP sequence; the pGEM-T-Easy-ΔeGFP containing the donor template for HR represented by a truncated wt EGFP lacking the first ATG codon and the sequence coding for the last 25 amino acids. This template contains an additional silent point mutation that abrogates the specific Cas9 PAM recognition site once HR has occurred. U2OS cells were reverse transfected 72 hr in advance with a library of 2,042 human microRNAs mimics (Dharmacon). By high content fluorescent microscope analysis, we quantified GFP+ cells corresponding to single-cell gene targeting events; immunofluorescence staining (red fluorescence) with a GFP-specific antibody recognizing both misfolded and normal GFP, allowing normalization for transfection efficiency. We identified 21 microRNAs mimics that significantly increased HR events compared to controls ($P<0.001$). Strikingly, we discovered that 10 of the top miRNAs in the screen belonged to two associated miRNA families sharing the same seed (the discovery is currently being patented). Interestingly, these microRNAs were equally efficient at enhancing HR when tested in a reporter U2OS eGFP (Y66S) stable cell line, co-transfected with pX330-U6-sgEGFP5-Chimeric_BB-CBh-hSpCas9 and pGEM-T-Easy-ΔeGFP donor template. Additionally, a positive effect on HR efficiency was also observed in cultures of primary neonatal rat cardiomyocytes, using the same reporter constructs adapted to an AAV6 vector system. While we are now investigating on the molecular mechanisms leading to enhanced efficiency of HR, these findings encourage us in pursuing microRNA delivery as a tool to achieve gene correction at clinically relevant levels.

21. dCas9 Epigenome Editing Suggests Histone Methylation Does Not Always Precede Target Gene Repression

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Distinct epigenomic profiles of histone marks have been associated with gene expression by genome-wide studies, but information regarding the causal relationship is still lacking. With the revolution of RNA-guided CRISPR/Cas9 we can engineer easy-to-use, highly specific and targetable modifying factors to a genomic locus and manipulate the local epigenome. In this study we investigated a broad collection of 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 FOG1). The regulators were evaluated in a common architecture of a catalytically inactive dCas9 and at a common HER2 gene promoter, and assayed for transcriptional repression and deposition of the expected epigenetic mark. Importantly, a dCas9-fusion for all tested epigenetic regulators was found that could down-regulate HER2 gene expression. The most potent repressor and depositor was found to be the N-terminal 45 residues of FOG1 [FOG1(1-45)]; we found that FOG1-dCas9-FOG1 repression was associated with targeted deacetylation and tri-methylation of H3K27 (H3K27me3). However, repression by dCas9 fusions was not always accompanied by deposition of the relevant histone mark. Our results suggest that so-called repressive histone modifications are not required for gene repression. We additionally tested fusions to the dCpf1 programmable binding protein to increase the number of targetable sites in the genome. However, none of the dCpf1 fusions affected HER2 gene expression, suggesting important differences between the RNA-guided dCas9 and dCpf1 platforms. Our data demonstrate that the easily programmable dCas9 toolkit allows precise control of the epigenome and that dCas9 fusions with epigenetic-modifying enzymes can be used to dissect the relationship between the epigenome and gene regulation.

22. Easy and Efficient Gene Editing of Mammalian Embryos Using rAAV Vectors - From Mouse to Monkey

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Delivering Cas9 (mRNA or protein) and sgRNA into mammalian zygotes for gene editing has greatly expedited the generation of genetically modified animals. However, it still relies on laborious and time-consuming procedures including microinjection, as well as specialized equipment and techniques that collectively incur substantial expenses and turnaround. We found that some rAAV serotypes can efficiently transduce pre-implantation mouse embryos *ex vivo*, which prompted us to explore replacing microinjection with a simple rAAV infection protocol to deliver Cas9 and sgRNA into mouse zygotes for gene editing. We found that the infected embryos had a high frequency of gene editing (all cells in almost all embryos). The mice that were generated from explanted embryos showed the expected genotype and phenotype, and successfully transmitted the gene editing events through the germline. Importantly, the high gene editing efficiency is not limited to a particular gene, and can be achieved using small-scale rAAV preparations that require only common laboratory equipment and techniques. Unwanted rAAV genome integration is not detectable using a genome-wide sequencing approach. Combining with a third rAAV vector carrying a donor template, we also achieved precise genetic modification through homology-directed repair in ~50% *ex vivo* embryos. Encouraged by the data obtained in mice, we tested this approach in non-human primate (NHP) embryos. We found that NHP zygotes are also permissive to rAAV infection, and that highly efficient gene editing (up to 100% in some embryos) is achievable. Currently, we are in the process of using this strategy to create NHPs modeling Canavan disease, an inherited lethal pediatric leukodystrophy, which will allow us to test gene therapy for this currently untreatable disease in NHPs. As the expected dates of birth of the explanted embryos are three weeks before this meeting, relevant data regarding editing efficiency and phenotype will also be presented if available. Our approach to animal modeling offers unparalleled ease and efficiency, thus significantly advancing the generation of genetically modified animal models and the use thereof to assess efficacy and safety of gene therapy. D.W., Y.Y. and Y.N. are Co-first authors; W.J, J.A.R.-P. and G.G. are Co-corresponding authors.

23. New Zinc Finger Nuclease Architectures for Highly Efficient Genome Engineering in Primary Cells at Large Scale with No Detectable Off-Target Effects

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Therapeutic genome engineering offers the prospect of treatments for diverse conditions via the single-step introduction of precise genetic changes into disease-relevant tissues or cells. A considerable challenge for such approaches, however, involves the need to achieve therapeutically adequate levels of on-target modification (often > 80%) while keeping off-target effects sufficiently if not undetectably low. Reflecting this, many published studies have not examined nuclease specificity in highly modified primary cells, instead focusing on systems that are less highly modified and/or more experimentally tractable such as tumor lines.

To address the issue of cleavage specificity, we have developed new zinc finger nuclease (ZFN) architectures and variants that globally suppress off-target cleavage by over 100-fold, relative to the standard ZFN framework, enabling very high levels of on-target activity with little or no detectable off-target cleavage. These new ZFNs were generated by substituting residues known or anticipated to contact the DNA phosphate backbone. Our studies proceeded in three stages. First, individual amino acid substitutions were made in ZFNs with known off-target sites and the new variants were screened for selective reduction in off-target cleavage. Next, individual substitutions were combined to identify sets of alterations that further enhanced on-target cleavage preference. When tested in a ZFN pair targeted to the erythroid enhancer of BCL11A, these alterations suppressed cleavage by >100 fold at known off-target sites. Importantly the original and variant pairs exhibited similar dose-dependent modification of BCL11A, indicating that the specificity was improved with virtually no decrease in on-target activity. Finally, original and variant ZFNs were submitted to unbiased genomewide specificity analysis using an oligonucleotide duplex capture assay, followed by screening of

candidate off-target sites for modification in ZFN-treated CD34 cells. This study identified no significant modification of off-target sites in cells treated with the variant ZFNs, with an assay sensitivity of ~0.1%. Critically this study was performed on samples generated under large scale transfection conditions and used high ZFN levels for both the capture and follow-up assays (resulting in 85% and 80% on-target modification, respectively).

In subsequent studies we have shown that the architectures and design strategies developed for BCL11A are portable to other ZFN pairs. In a parallel effort we have also demonstrated that optimization of ZFN delivery ratio (i.e. introducing the two ZFNs at a ratio other than 1:1) provides a simple, orthogonal strategy for further enhancing specificity. Other loci and cell types have also been modified at large scale with little or no detectable off-target cleavage. These results, coupled with the very high design density of the ZFN platform, raise the prospect of using ZFNs to deliver a highly, if not uniquely, specific cleavage event to virtually any chosen genomic base pair.

24. Sustained Tau Reduction via Zinc Finger Protein Transcription Factors as a Potential Next-Generation Therapy for Alzheimer's Disease and Other Tauopathies

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Microtubule Associated Protein Tau (MAPT) is implicated in the pathogenesis of several human neurodegenerative diseases, collectively referred to as tauopathies, including Frontotemporal Dementia and Parkinsonism Linked to Chromosome 17 (FTDP-17), Progressive Supranuclear Palsy (PSP), and Alzheimer's Disease (AD). The safety and efficacy of reducing endogenous tau for the treatment of these and other tau-related disorders is supported by a large body of genetic evidence, and both antibodies and antisense oligonucleotides targeting tau are at various stages of clinical development. While these approaches hold promise, they rely on periodic systemic administration or intrathecal infusions of the therapeutic agent for a patient's lifetime. In this study, we developed engineered Zinc Finger Proteins (ZFPs) to repress MAPT transcription, which could act to both reduce established tau pathology and confer the potential neuroprotective benefit of lowering endogenous tau levels. ZFPs designed to target the region surrounding the mouse MAPT Transcription Start Site (TSS) were fused to the human KRAB transcriptional repression domain (ZFP-TF) and tested for activity and specificity in mouse and human cell models. Initial screening of transiently-transfected ZFP-TFs in Neuro2A cells identified several proteins capable of reducing tau mRNA levels by 50% to >95% with saturating dose-response profiles. AAV9-mediated delivery of CMV-driven ZFP-TFs to mouse primary cortical neurons was well tolerated and resulted in similar levels of tau mRNA and protein reduction. We further evaluated the activity of the AAV9-delivered tau ZFP-TFs in the context of three well-characterized neuronal promoters (Synapsin1, CAMKIIα, and MeCP2) and found that each was capable of driving 90 - 99% tau reduction in primary cortical neurons with varying EC50s. Candidate lead ZFP-TFs underwent an extensive transcriptome-wide specify evaluation at the maximum deliverable dose in Neuro2A cells, primary human fibroblasts, and mouse cortical neurons; proteins with few to zero off-targets in all cell settings were identified and a mouse lead was chosen for in vivo testing. Direct stereotactic injection of AAV9-ZFP-TF vectors into the wild-type mouse hippocampus resulted in 80-90% reduction of tau mRNA and protein. Importantly, ZFP-TF expression and tau reduction levels were sustained at all examined short- and long-term endpoints, and we observed minimal to no changes in astrocyte (GFAP) and microglial (IBA1) levels out to six months after injection as measured by qRT-PCR and histology. Taken together, these results demonstrate that tau-targeted ZFP repressors are well tolerated in vivo, and support the further preclinical development of an AAV-delivered ZFP-TF as a therapy for tauopathies. Studies are ongoing to evaluate the efficacy of endogenous tau reduction in AD models.

Genome Editing and Integration Analysis in Metabolic and Endocrine Disorders

25. Nuclease-Mediated Allelic Exchange as a Therapeutic Strategy to Repair Recessive Compound Heterozygous Mutations: Proof-of-Concept in a New Mouse Model of Tyrosinemia

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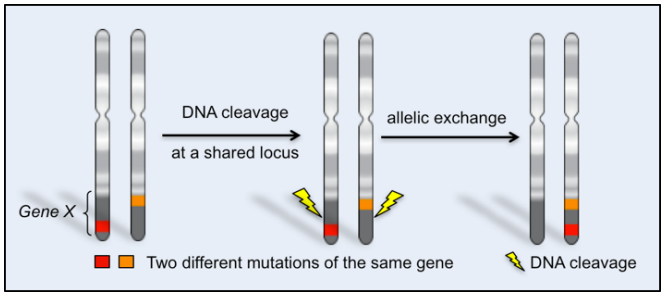
Patients suffering from monogenic recessive genetic disorders are often genotyped as compound heterozygous, which means the presence of two different loss-of-function mutant alleles of a particular gene. Here we report a novel approach to repairing such recessive compound heterozygous mutations by re-organizing the genetic material spread on a diploid genome to reconstitute a mutation-free allele. This is achieved by inducing double-stranded DNA breaks at the same location on both mutant alleles, followed by allelic exchange of genomic DNA **(Figure)**. We first proved the concept in a reporter mouse model, which carries $GFP^{N\text{-}term}$-intron-$tdTomato^{C\text{-}term}$ and $tdTomato^{N\text{-}term}$-intron-$GFP^{C\text{-}term}$ expression cassettes, respectively, at the same genomic locus on each copy of chromosome 11. Targeting the shared intron *in vivo* by rAAV9.Cas9-mediated DNA cleavage led to allelic exchange, and reconstituted the full-length GFP and tdTomato expression cassettes revealed by long-range DNA sequencing. Importantly, reporter-positive cells were detected in tissue sections by fluorescence microscopy, and quantified to be ~1% in liver and heart. To demonstrate the therapeutic efficacy of this approach, we crossed two homozygous mouse models of

hereditary tyrosinemia type 1 (HT1), which carry a neomycin insertion in exon 5 (E5) and a splicing mutation in exon 8 (E8) of the *Fah* gene, respectively. We treated the resulting compound heterozygous $Fah^{E5/E8}$ mice with rAAV vectors expressing Cas9 and sgRNA targeting an intron between the two mutation sites. The treatment yielded Fah-positive hepatocytes (by immunohistochemistry), and mutation-free mRNA (by reverse transcription-PCR) in the liver. Encouragingly, the treatment rescued the severe body weight loss as seen in the control mice, normalized liver histopathology (by hematoxylin and eosin staining) and diminished liver damage (by monitoring serum transaminase) in all treated animals. These data allowed us to conclude that the somatic allelic exchange approach successfully corrected the HT1-related phenotype in the compound heterozygous $Fah^{E5/E8}$ mice. The gene repairing approach described here is a novel strategy to correct recessive compound heterozygous mutations, a genotype category defining a large population of patients, especially of genetic metabolic diseases including HT1.



26. Rescue of Mice with Methylmalonic Acidemia from Immediate Neonatal Lethality Using an *Albumin* Targeted, Promoterless Adeno-Associated Viral Integrating Vector

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Methylmalonic acidemia (MMA) is rare genetic disorder most typically caused by mutations in methylmalonyl-CoA mutase (*MUT*), a mitochondrial localized metabolic enzyme. 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. However, metabolic instability can be eliminated by liver transplantation, which is used as a treatment for some patients. Conventional adeno-associated viral (AAV) mediated gene delivery is capable of effectively rescuing a neonatal murine model of MMA, reducing disease related metabolites, and improving growth. However, hepatocellular cancer was observed in a majority of the AAV-treated mice and was associated with AAV-mediated insertional mutagenesis at the *Rian* locus. To reduce the risk of AAV mediated genotoxicity and to increase the longevity of MUT expression, we employed AAV delivery of a targeted-integrating-promoterless vector carrying the *MUT* gene. This novel vector utilizes homologous recombination for site-specific gene addition of human *MUT* into the mouse albumin (*Alb*) locus immediately upstream of mouse *Alb* stop codon. Our promoterless AAV vector, AAV-Alb-A2-MUT, contains a 2A-peptide coding sequence proximal *MUT* gene, which allows for *MUT* expression by the endogenous *Alb* promoter. We have previously reported that AAV8 delivery of a dose of 2.5E12 Alb-A2-MUT vg/pup at birth reduced disease related metabolites and increased growth in a hypomorphic murine model of MMA. Now we demonstrate that delivery of an AAVDJ vector at a dose of 8.6E11 Alb-A2-MUT vg/pup at birth rescues the neonatal lethal phenotype exhibited by complete *Mut* knock-out mice ($Mut^{-/-}$). Two of the three rescued AAVDJ-Alb-A2-MUT treated $Mut^{-/-}$ mice are still alive three months after treatment but are smaller than their wild-type littermates. Rescuing a murine model that displays an immediate neonatal-lethal phenotype is one of the most challenging gene delivery experiments to execute successfully. Hence our study, although small in size, provides the first demonstration that targeted genome editing into *Albumin* has the potential to be therapeutic for neonatal forms of MMA, and sets a standard for vector efficacy related to this approach.

27. Liver-Based Expression of the Human alpha-Galactosidase A Gene in a Murine Fabry Model Results in Continuous High, Therapeutic Levels of Enzyme Activity and Effective Substrate Reduction

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Fabry disease is an X-linked lysosomal storage disease caused by mutations in the GLA gene encoding the enzyme alpha galactosidase A (α-GalA) and the progressive systemic accumulation of its primary substrate, globotriaosylceramide (Gb3/GL-3), leading to renal, cardiac and cerebrovascular disease, with reduced life expectancy. Depending on the mutation and residual α-GalA enzyme level the disease presents as classic early-onset Fabry in childhood/adolescence or as an attenuated form later in life.

In both cases the disease is commonly treated by enzyme replacement therapy (ERT). However, ERT requires a lifetime of biweekly infusions and may not clear all substrate from secondary organs. Therefore the development of a one-time treatment for Fabry with improved and long-lasting effectiveness is desirable. Two AAV-mediated, liver-targeted gene therapy approaches were evaluated in a knock-out mouse model for Fabry (GLAKO) that has no α-GalA activity and accumulates high levels of Gb3 (and its soluble form lyso-Gb3) in plasma and tissues.

The first approach uses an episomal AAV vector encoding a codon-optimized version of the human GLA cDNA (hGLA) driven by a liver-specific promoter. This vector was administered to GLAKO mice followed by weekly monitoring of plasma α-GalA activity. Supraphysiological enzyme levels were achieved as early as day 14 and sustained throughout the 8 week length of the study. Tissue analyses at study end demonstrated a dose-dependent increase in α-GalA activity

in the liver, heart, kidney and spleen along with a corresponding reduction in Gb3/lyso Gb3 levels. Gb3 was undetectable in the tissues of some GLAKO mice administered high dose hGLA cDNA.

The second strategy (currently in clinical studies for the treatment of Hemophilia B and Mucopolysaccharidosis types I & II) uses ZFN-mediated genome editing to permanently modify GLAKO murine liver cells by inserting a corrective copy of the hGLA gene in the Albumin locus. This system exploits the high transcriptional activity of the endogenous Albumin enhancer/promoter and uses stably modified hepatocytes to potentially allow long-term expression of the transgene. For this approach three AAV vectors (two ZFN vectors and one hGLA cDNA donor vector) were administered followed by weekly monitoring of plasma α-GalA activity. This in vivo protein replacement strategy achieved supraphysiological levels of plasma α-GalA activity that were sustained for the duration of the 8 week study. High levels of α-GalA activity were detected in the liver, heart, kidney and spleen of treated mice. Gb3 content in these tissues averaged less than 10% of the storage measured in untreated GLAKO mice.

The results of both studies will be compared and analyzed for establishment of the necessary long-term α-GalA levels in vivo. These findings provide "proof-of-concept" for using AAV-mediated targeting of hepatocytes to express therapeutic levels of human α-GalA, resulting in marked reduction of the accumulated Gb3/lyso Gb3 in key tissue sites of pathology.

28. Amelioration of Alpha-1 Antitrypsin Deficiency Diseases with Genome Editing in Transgenic Mice

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Alpha-1 Antitrypsin Deficiency (AATD) is a hereditary liver and lung disease caused by mutations in the SERPINA1 gene. Most patients with severe clinical manifestations are homozygous for PiZ alleles with E342K mutations, characterized by aggregation of misfolded protein in hepatocytes and insufficient secretion of AAT into circulation. Liver injury due to protein aggregation leads to inflammation, cirrhosis and hepatocellular carcinoma. The absence of circulating AAT allows unchecked neutrophil elastase activity to damage the lungs leading to emphysema. In this study, we demonstrate correction of the AATD liver phenotype in the human PiZ transgenic mouse model using two different genome editing approaches. The first approach utilized systemic delivery of a single AAV-CRISPR targeting Exon II of human SERPINA1. The second approach employed a dual-AAV system to correct the nucleotide responsible for the Z mutation in the transgenic mice via homology-dependent repair. The predominant outcome of both approaches was a significant reduction (up to 99%) in circulating levels of human AAT-Z as well as reduced AAT-Z aggregate formation (up to 88%) in the livers of treated mice as shown by ELISA and histology, respectively. Moreover, the dual-AAV approach resulted in 5% correction of the PiZ mutation in adult PiZ mice as assessed by RNAseq. In conclusion, CRISPR/Cas9-mediated gene editing technologies represent a promising avenue for a potential one-time treatment for AATD patients.

29. Somatic Genome Editing with AAV Vectors Generates and Corrects a Metabolic Disease

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The Clustered Regularly-Interspaced Short Palindromic Repeats/Cas9 (CRISPR/Cas9) genome editing system holds great promise for the study as well as treatment of monogenic disorders. We hypothesized that Adeno-Associated Viral (AAV) vectors could be used to deliver guide RNAs (gRNA) to somatically disrupt the low density lipoprotein receptor (*Ldlr)* gene in adult Cas9 transgenic mice as a model of Familial Hypercholesterolemia. We also hypothesized that editing could be multiplexed to test the involvement of other liver-expressed candidate genes, using the therapeutic target *Apob* as a proof-of-principle. Mice were injected with AAV8 vectors encoding either *1)* a nontargeting gRNA, *2)* a gRNA targeting *Ldlr*, or *3)* gRNAs to *Ldlr* and *Apob*, and placed on Western diet for twenty weeks. Disruption of *Ldlr* with AAV-CRISPR was robust, resulting in severe hypercholesterolemia (728 +/- 174 mg/dl) and atherosclerotic lesions in the aorta. Mice receiving gRNAs to both *Ldlr* and *Apob* had an identical degree of *Ldlr* disruption, but dramatically lower plasma cholesterol levels (125 +/- 27.3 mg/dl), profound hepatic steatosis, and complete protection from atherosclerosis. We observed a low, but detectable frequency of off-target editing for the *Ldlr* gRNA, and none for the *Apob* gRNA at predicted sites. Interestingly, we also observed integration of the viral Inverted Terminal Repeat (ITR) sequences at one of the editing sites, highlighting an additional safety consideration for AAV-based genome editing. This approach can be used to study atherosclerosis and other metabolic liver diseases, and our work provides valuable information about the safety and specificity of liver-directed genome editing.

30. ZFN-Mediated *In Vivo* Genome Editing Results in Phenotypic Correction in MPS I and MPS II Mouse Models

Russell DeKelver[1], Li Ou[2], Kanut Laoharawee[2], Susan Tom[1], Robert Radeke[1], Michelle Rohde[1], Scott Sproul[1], Michael Przybilla[2], Brenda L. Koniar[2], Kelly M. Podetz-Pedersen[2], Renee D. Cooksley[2], Michael C. Holmes[1], R. Scott McIvor[2], Chester B. Whitley[2], Thomas Wechsler[1]

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Mucopolysaccharidosis types I & II result from the deficiency of lysosomal enzymes α-L-iduronidase (IDUA) and iduronate 2-sulfatase (IDS), respectively, and the subsequent systemic accumulation of glycosaminoglycans (GAGs), leading to severe morbidity and a shortened lifespan in patients. Although the severe form of MPS I (Hurler syndrome) can be treated by hematopoietic stem cell transplantation, this procedure carries significant morbidity and mortality risks. Additional treatment for MPS I & II consists of enzyme replacement therapy, which slows disease progression but requires lifelong weekly infusions and can be a severe hardship on patients.

We have developed a ZFN-mediated genome editing strategy to permanently modify patient liver cells through insertion of a corrective hIDUA or hIDS gene at the Albumin locus. 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 of the corrective transgene 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.

In MPS I and MPS II mouse models, following treatment with AAV2/8 vectors comprising the Albumin-targeting ZFNs and the respective corrective human transgene donor, we demonstrate supraphysiological hIDUA or hIDS enzyme levels in the liver, secretion of active enzyme into the plasma, and efficient uptake by secondary tissues at levels sufficient for the complete clearance of GAG substrate. Histological observations demonstrate reduced disease-related cellular vacuolation, further supporting reduced GAG accumulation. ZFN+Donor treatment also prevented the emergence of cognitive deficits exhibited by both animal models in the Barnes maze at 4 months post-treatment, in which the treated animals had behavior similar to wild type mice.

Biochemical characterization of the hIDS and hIDUA proteins expressed from the inserted transgenes demonstrate the expected glycosylation patterns and mannose-6-phosphate-dependent cellular uptake in vitro.

In summary, our data provide proof-of-concept for ZFN-mediated targeting of the Albumin locus in hepatocytes as an in vivo protein replacement platform to express therapeutic amounts of hIDUA and hIDS for the potential treatment of MPS I & II.

31. Molecular Characterization of Hematopoietic System Reconstitution in 7 Metachromatic Leukodystrophy Patients Following Hematopoietic Stem Cell Gene Therapy

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Here we report the in-depth molecular monitoring of hematopoiesis of the first 7 patients enrolled in a self-inactivating lentiviral vector (LV) -based clinical trial for metachromatic leukodystrophy (MLD) conducted at SR-Tiget (up to 6 years after treatment). Using standard Linear Amplification Mediated (LAM) PCR and quantitative method for integration site (IS) retrieval and improved bioinformatics pipelines we analyzed the LV integration profile in CD34+ cells, multiple myeloid and lymphoid cell markers purified from bone marrow and/or peripheral blood harvested from each patient at different time points after therapy. From each patient were retrieved >10.000 IS suggesting a polyclonal reconstitution and confirmed by Shannon diversity analysis. The reconstitution of the hematopoietic system in terms of population diversity showed similar pattern for all patients, with an initial fluctuation period in clonal diversity, 1-9 months, followed by a stabilization period from 12-18 months. As reported previously vector marking in lymphoid cells in blood is at first oligoclonal and turns polyclonal after 6 months. Myeloid cells instead were highly polyclonal already at the first time points. Clonal abundance analysis did not show any evidence of clonal dominance. To study HSPC activity during time we performed mark and recapture statistics on IS belonging to short lived cell compartments recaptured over time points. By this analysis we observed that the number of HSPC at earlier time point present the highest activity that progressively stabilize to ~10.000 from 9 months post transplantation. These data suggest that the initial waves of repopulation are sustained by short lived progenitors. Finally IS were shared among HSPC and differentiated cells indicating that multilineage reconstitution of genetically modified cells has occurred. Interestingly, in all patients and in agreement with our previous observations there is a progressive increase in the relative percentage of IS with multilineage potential until 12-18 after transplant, after that point however the multilineage potential drastically and stably decreased. These results, analyzed by a multivariate statistics model for heterogeneity testing do not appear to be arising from confounding variables such as cell purity (>95%) or vector copy number (VCN=1) and others.Importantly these data suggest that hematopoiesis after transplantation is sustained by short lived progenitors that are progressively lost while a period of intense HSC multilineage activity is mounting until 18 months after transplant to turn in lineage committed hematopoiesis, reminiscent to the recently described mechanism of homeostatic maintenance of normal hematopoiesis by committed progenitors.

32. Effect of AAV Gene Therapy on Hepatic Tumor Induced by Deficiency of Glucose-6-Phosphatase

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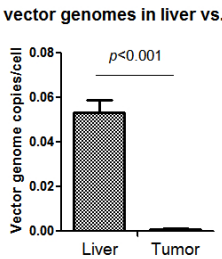
Glycogen storage disease type Ia (GSD Ia) is a rare inherited disease caused by mutations in glucose-6-phosphatase (G6Pase) catalytic subunit gene (*G6PC*). G6Pase deficiency causes life-threatening hypoglycemia treated by a strict diet to maintain normal blood glucose. GSD Ia patients still develop long-term hepatic complications, which include steatohepatitis and development of hepatic adenomas with a risk for hepatocellular carcinoma. Our previous study showed that use of a zinc-finger nuclease (ZFN) targeted to the ROSA26 safe harbor locus and a ROSA26-targeting vector containing a *G6PC* donor transgene, both delivered with adeno-associated virus (AAV) vectors, markedly improved survival in the *G6pc*(-/-) mouse model for GSD Ia. Based on these results, we designed experiments to determine if genome editing can suppress hepatic tumors in liver-specific *G6pc*(-/-) mouse model (L- *G6pc*-/-). Here we present that administration of vectors containing both the ZFN and *G6PC* donor to adult L- *G6pc*-/- mice achieved decreased glycogen content in liver, in comparison with ZFN ($p<0.001$) or G6PC alone ($p<0.01$). Administration of both the ZFN and *G6PC* donor increased G6Pase activity in liver, in comparison with ZFN alone ($p<0.05$), which correlated with higher blood glucose for up to 12 months of age ($p<0.001$). These data indicate that genome editing with vectors containing ZFN and *G6PC* donor work efficiently in the L- *G6pc*-/- model for GSD Ia. Moreover, the ZFN and *G6PC* donor treated mice had fewer liver tumors, and a lower number of tumors per mouse, although these differences were not statistically significant. Interestingly, we found that vector genomes were almost undetectable in the tumors, and remained relatively high in adjacent liver (Fig). These data suggested that expression of G6Pase by genome editing can correct the liver abnormalities of GSD I, which may suppress tumorigenesis. To verify whether the same phenomenon exists in the canine GSD I model, we analyzed the liver and tumor samples in AAV vector-treated dogs with GSD Ia. We found that tumors showed lower G6Pase activity ($p<0.01$), in comparison with adjacent liver in vector treated dogs. Furthermore, the vector-treated dog liver had elevated G6pase activity ($p<0.01$), compared to untreated GSD Ia dog controls. Taken together, these results suggest that AAV-mediated gene therapy not only corrects hepatic G6Pase deficiency but also has potential to suppress tumor formation in the GSD Ia liver.



Gene Therapies for Musculoskeletal Diseases

33. Systemic Gene Editing for Muscular Dystrophy Using AAV-CRISPR/Cas9

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AAV-mediated dystrophin gene editing strategies using the CRISPR/Cas9 system have been shown to be capable of inducing dystrophin expression in mouse models of Duchenne muscular dystrophy (DMD). However, therapeutically relevant dystrophin expression levels have only 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 the dose needed for near complete transduction and transgene expression in all muscle groups, optimal doses 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 gRNA expression cassettes often need to be separated into two different vectors for co-delivery, which increases the effective 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 (the ratio of corrected vs. non-corrected genomes) after target cell transduction, a limitation not present for gene replacement studies. Here we present *in vivo* approaches for muscle-specific correction of the dystrophin gene in the *mdx*^{4cv} mouse model of DMD using multiple AAV vector delivery systems. Muscle-restricted Cas9 expression enables direct editing of the mutation, multi-exon deletion or complete gene correction via homologous recombination in myogenic cells. Treated muscles demonstrate widespread dystrophin expression and increased force generation following intramuscular delivery. Systemic gene editing using muscle-restricted Cas9 expression results in widespread expression of dystrophin in both skeletal and cardiac muscles, with the added benefits of improved specificity and safety. Overall, AAV-mediated muscle-specific gene editing shows significant potential for permanent correction of mutations leading to DMD and other genetic muscle disorders.

34. 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 characterized by progressive muscle wasting, loss of ambulation, and death typically occurs in the third decade of life due to respiratory and cardiac complications. DMD is also the most common fatal genetic disease. The disease results from deleterious mutations in the dystrophin gene that disrupts the translational reading frame and cause a loss of functional dystrophin protein. Becker muscular dystrophy (BMD) is similar to DMD in that it results from deletions in the dystrophin gene. However, the BMD deletions maintain the translational reading frame and result in the production of internally truncated but partially functional dystrophin protein. The BMD phenotype is often much less severe than DMD, and thus converting DMD to a BMD genotype by restoring the dystrophin reading frame is a commonly explored therapeutic strategy. CRISPR/Cas9 is a genome engineering tool that can be used to target specific loci to create precise changes in DNA sequences. We have previously utilized *S. pyogenes* Cas9 (SpCas9) to restore dystrophin protein expression in immortalized myoblasts from DMD patients by targeting the intronic regions surrounding exon 51 to repair the disrupted reading frame. Furthermore, we have previously applied the smaller *S. aureus* Cas9 (SaCas9), which is compatible with AAV packaging restrictions, to remove mouse exon 23 in the mdx mouse model and restore dystrophin expression and muscle function *in vivo*. This work showed proof-of-principle of a CRISPR-based gene therapy for DMD, but ultimately CRISPR/Cas9 systems that target the human dystrophin gene must be evaluated in animal models. Thus, we have continued this work by developing an SaCas9 system targeted to the intronic regions around exon 51 in the human dystrophin gene. gRNAs were screened for activity levels in vitro in HEK293T cells as well as immortalized myoblasts from DMD patients. The expected deletion of exon 51 was confirmed by end point PCR and quantified by digital droplet PCR of the genomic DNA and the dystrophin cDNA. Western blot of protein lysates from treated DMD myoblasts in differentiation culture confirmed the restoration of dystrophin expression using this system. To test these human-targeted gRNAs *in vivo*, we developed a novel dystrophic mouse model in which exon 52 has been removed from the human gene, creating a disrupted reading frame that is correctable by deletion of exon 51. We confirmed the dystrophic phenotype of these mice biochemically and functionally by activity and grip strength assays. *In vivo* delivery of the new CRISPR system by intravenous administration of AAV vectors resulted in restored human dystrophin expression in skeletal and cardiac muscle tissues, demonstrated by PCR and sequencing of the DNA and mRNA, western blot, and immunohistochemistry. Moreover, functional benefit was demonstrated compared to untreated mice in both activity and muscle strength assays. This work is significant in extending the early proof-of-principle studies to a translational strategy for gene editing as a potential treatment for DMD.

35. Efficient In Vivo Correction of a Splicing Defect Using an HDR-Independent Mechanism

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According to recent estimates, splice site defects account for up to 10% of pathogenic mutations causing Mendelian diseases. The prevalence is even higher for neuromuscular disorders (NMDs) due to the unusually large size and multiexonic structure of genes encoding muscle structural proteins, further highlighting the importance of these mutations in NMDs. Therapeutic genome editing can be exploited to correct disease-causing mutations. However, in previous studies, correction of splice site mutations has only been accomplished via the homology-directed repair (HDR) pathway, which is extremely inefficient in post-mitotic tissues such as skeletal muscles, hampering its therapeutic utility in NMDs. Here, we demonstrate a novel strategy to correct of a pathogenic splice site mutation in NMDs by harnessing the non-homologous end-joining (NHEJ) repair pathway. As a proof-of-principle, we focus on merosin-deficient congenital muscular dystrophy type 1A (MDC1A), which is characterized by severe muscle wasting and paralysis. Specifically, we correct a splice site mutation in the Lama2 gene, which causes exclusion of exon 2 and truncation of Lama2 protein in dy^{2J}/dy^{2J} mouse model of MDC1A. Using adeno-associated viral vector serotype 9 (AAV9) to deliver S. aureus Cas9 and two guide RNAs, we simultaneously excise intronic region containing the mutation to create a functional splice donor site through NHEJ. This strategy leads to successful inclusion of exon 2 in the Lama2 transcript and restoration of full-length Laminin-α2 protein. Importantly, the treated dy^{2J}/dy^{2J} mice display significant improvement in muscle histopathology, strength and function without any signs of paralysis. Our results demonstrate an innovative approach to correct a non-coding mutation by modifying an intronic region to create a functional splice donor site that is independent of the HDR pathway. Given that a significant proportion of MDC1A individuals are affected by splice site mutations, this strategy carries a therapeutic potential for numerous patients. Furthermore, it highlights a far-reaching therapeutic potential and translatability of this strategy for diseases caused by splice site mutations.

36. Systemic Delivery of Dysferlin Overlap Vectors Provides Long-Term Functional Improvement in Dysf$^{-/-}$ Mouse Model of LGMD2B

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Dysferlinopathies comprise a family of disorders caused by mutations in the dysferlin (DYSF) gene leading to a progressive dystrophy characterized by chronic muscle fiber loss, fat replacement and fibrosis. To correct the underlying histopathology, expression of full-length DYSF is required. We have developed dual adeno-associated virus vectors defined by a region of homology to serve as a substrate for reconstitution of the full 6.5 kb dysferlin cDNA. Our previous work studied the efficacy of this treatment through intramuscular and regional delivery routes. To maximize clinical efficacy, we treated dysferlin-deficient mice systemically to target all muscles through the vasculature for efficacy and safety studies. Mice were systemically dosed with $6x10^{12}$vg and evaluated at various timepoints between 3 and 15 months post-treatment for dysferlin expression, morphological changes and functional improvement using MRI, MR-spectroscopy and membrane repair. The systemic dose of $6x10^{12}$vg resulted in widespread gene expression in muscle. Treated muscles showed a significant decrease in central nucleation, collagen deposition and improvement of membrane repair to wild-type levels. Treated gluteus muscles were significantly improved versus placebo treated muscles and were equivalent to wild-type in volume, intramyocellular and extramyocellular lipid accumulation and fat percentage using MRI/MRS. Findings in mice showed clear evidence that functional dysferlin was successfully delivered. Additional studies in non-human primates further confirmed dysferlin expression. Our dual-vector treatment allows for production of functional full-length dysferlin with no evidence of toxicity seen in either animal model. This confirms previous safety data and further validates translation to dysferlinopathy patients.

37. Systemic Injections of Peptide-Conjugated Morpholinos Improve Cardiac Symptoms of a Dog Model of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a lethal genetic disorder caused by an absence of dystrophin protein in body-wide muscles, including the heart. Cardiomyopathy is a leading cause of death in DMD. Exon skipping via synthetic phosphorodiamidate morpholino oligomers (PMOs) represents one of the most promising therapeutic options, yet PMOs have shown very little efficacy in cardiac muscle. To increase therapeutic potency in cardiac muscle, we tested a next-generation morpholino - arginine-rich, cell-penetrating peptide-conjugated PMOs (PPMOs) - in the canine X-linked muscular dystrophy in Japan ($CXMD_J$) dog model of DMD. A PPMO cocktail designed to skip *dystrophin* exons 6 and 8 was injected intramuscularly, intracoronarily, or intravenously into $CXMD_J$ dogs. Intravenous injections of PPMOs restored expression of dystrophin protein in the myocardium and cardiac Purkinje fibers, as well as skeletal muscles. Vacuole degeneration of cardiac Purkinje fibers, as seen in DMD patients, was ameliorated in PPMO-treated dogs. Electrocardiogram abnormalities (increased Q amplitude and Q/R ratio) were also improved in $CXMD_J$ dogs after intracoronary or intravenous administration. No obvious evidence of toxicity of PPMOs was found in blood tests throughout the monitoring period. The present study is the first to report rescue of dystrophin expression and recovery of the conduction system in the heart of dystrophic dogs by PPMO-mediated multi-exon skipping, and demonstrates the high clinical potential of systemic PPMO therapy for cardiac symptoms of DMD.

38. Evaluation of Re-Administration of a Recombinant Adeno-Associated Vector Expression Acid Alpha-Glucosidase (rAAV9-DES-hGAA) in Pompe Disease: Preclinical to Clinical Planning

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A recombinant serotype 9 adeno-associated virus (rAAV9) vector carrying a transgene that expresses codon optimized human acid alpha-glucosidase (hGAA, or GAA) driven by a human desmin (DES) promoter (i.e. rAAV9-DES-hGAA) has been generated as a clinical candidate vector for Pompe disease. The rAAV9-DES-hGAA vector is being developed as a treatment for both early and late onset Pompe disease, in which patients lack sufficient lysosomal alpha-glucosidase leading to glycogen accumulation. In young patients, the therapy may need to be re-administered to maintain therapeutic levels of GAA. To establish the basis for re-administration of AAV vectors we have completed IND-enabling pre-clinical studies testing immune reactions after the concomitant use of immune modulation with local and systemic delivery of AAV9. Both mouse and NHP data were obtained to establish the safety of this approach. GAA-/- mice were engineered to also express human CD20 and therefore amenable to use of human anti-CD20 antibody treatment. The immune modulation regimen is based on non-clinical and clinical data supporting B-cell ablation with rituximab and co-administration of sirolimus immediately

prior to vector exposure. Data from the NHP study showed that 1) Biodistribution of AAV9 is not affected by immune modulation. 2) Sirolimus or rituximab alone were not as effective as the combination of sirolimus and rituximab due to the potentiation of the B-cell depletion by both compounds, 3) Protection from anti-AAV antibodies allowed for re-administration of AAV9 given IM, 4) Screening for naïve status required the assessment of both total antibody titer by ELISA as well as neutralizing Ab titer, 5) immune modulation was able to completely block anti-human transgene immune response in NHPs, and 6) in the setting of pre-immunity, high-titer anti-AAV antibodies can lead to severe infusion reactions when AAV is delivered systemically. Findings from human CD-20+/KO mice confirmed the above findings and showed that repeated IV dosing leads to augmentation of GAA activity above wildtype levels without increased anti-GAA or anti-AAV9 antibodies from baseline levels. In conclusion, co-administration of rituximab and sirolimus with AAV vectors does not impact AAV biodistribution and is safe in non-human primates. In naïve animals, blockade of humoral immunity can prevent anti-AAV and anti-transgene antibodies which enhances safety and allow for repeated dosing of the therapeutic vector.

39. Prolonged Exon 2 Skipping and Robust Dystrophin Expression 1 Year Post Single Neonatal Injection of an AAV9.U7snRNA Vector in the Dup2 Mouse

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Mutations that truncate the reading frame 5' of exon 5 of the *DMD* gene result in use of an internal ribosome entry site (IRES). This element allows alternate translational initiation beginning within exon 6 that results in expression of an N-truncated isoform. Despite lacking half of the actin binding domain 1, this isoform is highly functional, as demonstrated by the minimal symptoms in patients who express it. We developed an AAV9.U7snRNA vector directed against exon 2 which induces skipping of this exon, thus resulting in a truncation of the reading frame therefore forcing expression of the highly functional N-truncated protein. injection of this vector have shown that in a Duchenne muscular dystrophy (DMD) mouse model carrying a duplication of exon 2 (the Dup2 mouse), postnatal intramuscular (IM) or late or early intravascular (IV) treatment results in functional and pathologic improvement in skeletal muscle. Of importance, earlier delivery of the vector result in almost complete protection of the muscle 6months post injection which is highly relevant to efforts to identify and treat DMD patients at an earlier age. Here we are presenting a follow up study one year post injection. Dup2 mice were injected via facial vein at postnatal day 1 (P1) with 1E12 total vector genomes of the AAV9.U7snRNA vector and sacrificed at either 12 months post-injection for evaluation of exon 2 skipping by RT-PCR, quantification of dystrophin expression, and characterization of histopathology. To model the applicability of this approach beyond exon 2 duplication patients, the same vector was used to treat 6 human patient fibroblast-derived transdifferentiated myoblasts (FibroMyoD cells) harboring various mutations within exons 1 to 4. In the Dup2 mouse, efficient skipping and abundant dystrophin expression were still present at one year following the single AAV injection. Dystrophic pathology was absent at all-time points; at one year, less than 1 % of fibers showed central nucleation, in comparison to ~70% in untreated Dup2 mice. Two tests on the ex vivo diaphragm preparations: isometric force (providing assessment of strength), and eccentric contractions (evaluating sarcolemma stability) were performed at 12 months following P1 injection. Both tests demonstrated a significant protection of the treated animals compare to the untreated. In all FibroMyoD cultures, abundant exon 2 skipping and dystrophin expression were detected in myotubes at 14 days of culture after treatment. These results suggest that this exon-skipping vector offers a therapeutic approach not only to patients with exon 2 duplications but with all mutations within the first four *DMD* exons (~6% of patients), an area of the gene largely ignored by the current therapeutic approaches. This work strongly supports the idea that early treatment of these patients will have longstanding and significant benefit resulting in a better outcome.

40. Toxicity & Biodistribution Study Demonstrates Safety of AAV-Mediated Human Beta-Sarcoglycan Gene Therapy

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Limb-girdle muscular dystrophy type 2E (LGMD2E) results from mutations in the beta-sarcoglycan (SGCB) gene causing loss of a sarcolemmal structural protein component of the dystrophin-associated protein complex (DAPC). This leads to a progressive dystrophy resulting in deteriorating limb and diaphragm muscle function, respiratory failure, and cardiomyopathy in 50% or more of LGMD2E patients. To investigate human SGCB (hSGCB) gene transfer to treat skeletal and cardiac muscle deficits, we designed a self-complementary (sc) AAVrh74 vector containing a codon optimized hSGCB transgene driven by a muscle specific promoter. We demonstrated efficacy of regional vector delivery by intramuscular (IM) injection and isolated-limb perfusion (ILP) to the lower limb muscles. We then delivered scAAV.hSGCB through the tail vein of SGCB-/- mice to provide a rationale for a clinical trial that would lead to clinically meaningful results. Tail vein injection of scAAV.hSGCB resulted in 98.13% transgene expression across all skeletal muscle which was sustained up to at least 15 months post-injection. This was accompanied by improvements in histopathology, kyphoscoliosis, diaphragm force production, creatine kinase levels, and overall activity. As a next step in the clinical development of hSGCB gene therapy, we performed a comprehensive toxicity study in wild-type (WT) mice to assess any potential safety concerns of hSGCB gene therapy. Utilizing a stepwise approach, cohorts of C57BL6 WT mice were treated by IM injection ($1x10^{13}$ vg/kg) or by ILP using a 2-dose escalation (low: $2x10^{12}$ vg/kg; high: $2x10^{13}$ vg/kg) and randomized into 6 and 12 week endpoints. Finally, cohorts of C57BL6 WT and SGCB-/- mice were treated by intravenous delivery of scAAV.hSGCB at $5x10^{13}$ vg/kg. Animals injected with lactated ringers solution served as control cohorts. Mice

in the different cohorts were subject to clinical monitoring, blood chemistry and antibody analyses, and endpoint measures including histopathology and vector genome and protein biodistribution. In-life clinical monitoring of animals from all treatment groups revealed no treatment-related clinical observations. No circulating antibodies to hSGCB were detected by ELISA (<1:25) and antibody responses to AAVrh74 increased following exposure with median endpoint titers ranging from 1:3200 to 1:6400. No test article-related changes were observed in the hematology and serum chemistry parameters, and no microscopic changes in tissue histopathology were observed in any vector dosed mice. While hSGCB vector genomes were detected in most tested tissues (>1000 copies/μg total DNA), no off-target expression of hSGCB was observed in any non-muscle tissue. We report that delivery of an AAV-mediated hSGCB gene therapy to WT and SGCB-/- mice has demonstrated no significant toxicity or safety concerns. In this well-defined model of LGMD2E, we have now shown efficacy and safety of AAV-mediated gene therapy, establishing a path forward to the clinical application of our therapy for LGMD2E patients, one we are currently pursuing. A phase I systemic safety trial is currently planned for LGMD2E patients.

Synthetic/Molecular Conjugates and Physical Methods of Gene Delivery I

41. Ultrasound Mediated Gene Delivery of High Expressing Factor VIII Variant Plasmids Combined with Immunomodulation Generated Long Term Therapeutic Levels of Factor VIII in Hemophilia A Mice

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Gene therapy offers great promises for a cure of hemophilia A disease that resulted from a deficiency of a single gene encoding the blood coagulation factor VIII (FVIII). Our lab has demonstrated that ultrasound (US)-mediated gene delivery (UMGD) can significantly enhance gene transfer efficiency of reporter plasmids. Previously we also reported that using a semi-focused US transducer, H158, average of ~15% of FVIII can be achieved on day 1 following gene transfer of a liver-specific (LC)-FVIII plasmid into hemophilia A mice. However, an anti-FVIII immune responses ensued significantly decreased FVIII levels in circulation. In order to achieve persistent FVIII gene expression for therapeutic treatment, several improvements are pursued. First, we incorporate a new novel FVIII variant with 10 amino acid substitutions in the A1 domain of the FVIII heavy chain (FVIIIX10, kindly provided by Weidong Xiao) into the liver-specific vector to generate LC-FVIIIX10. By hydrodynamic injection of the naked plasmid, LC-FVIIIX10 generates more than 10 fold higher FVIII expression in circulation compared with our original LC-FVIII/N6 plasmid. Second, we found recently that therapeutic UMGD pressure threshold was lowered by increasing pulse durations. By employing pulse-train US exposure with acoustic pressure of 1.0 MPa and pulse duration of 0.4 ms, comparable or higher transgene expression is achieved compared with those generated by continuous US exposure at higher pressures (2.0 and 2.7 Mpa and pulse duration of 0.018ms). Most significantly, we observe less transient liver damage evaluated by transaminase levels and histological examination. Third, in order to overcome anti-FVIII immune responses, two groups of the hemophilia A mice are treated separately with immunomodulation regimens using either dexamethozone for 5 days (day 1 - 5) to reduce innate and adaptive responses and IL2/IL2mAb complexes (at days -6, -5, -4, -1) to induce expansion of regulatory T cells and suppress T helper cell function or dexamethasone for 10 days (day 1- 10) and IL2/IL2mAb complexes (at days -6, -5, -4, -1, 2, 3, 4). Control group of mice is treated with UMGD but without immunomodulation. Following UMGD, all groups of mice yield 25- 100% of FVIII on day 1- 7. In control mice without immunomodulation, FVIII levels drop to undetectable at day 14 and afterwards with the formation of high-titer anti-FVIII inhibitory antibodies (50-120 BU). In the group of mice with shorter duration of immunomodulation, there is a delay in the formation of low titer antibodies (~1 BU) at day 14 and 21, however the titer is increased at later time points. In the group of mice with longer duration of immunomodulation, no inhibitory antibody is observed in the treated mice thus far (experimental duration) and the FVIII expression remains at therapeutic levels (10-50%). Phenotypic correction of hemophilia A mice is examined by tail clip assay. These exciting results demonstrate that UMGD can achieve safe and effective treatment of hemophilia A.

42. Plasmid Delivery to the Skin Using Increased Temperature and Gene Electrotransfer

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The easy accessibility of skin makes it an excellent target for gene therapy applications. Gene electrotransfer (GET) of skin is a simple, direct, *in vivo* method to deliver genes for therapy and can be accomplished in a minimally invasive way. We have previously demonstrated that GET can be used to effectively deliver plasmid DNA in several animal models with varying skin thickness and have demonstrated various potential applications including wound healing, treatment of peripheral ischemia and delivery of DNA vaccines. Part of this development has included newly designed electrode arrays specifically for skin-based applications and that could be easily applied and expanded. The limitations that we have encountered with our new designs are related to the depth of penetration of expression and occasionally cellular or tissue damage due to the required high applied voltage. GET requires a balance between efficient delivery and the maintenance of cell viability. In this current study, we have evaluated the addition of externally applied thermal energy to GET protocols to enhance delivery without increasing the applied voltage. We have determined both in vitro and in vivo that elevating the tissue temperature to between 42-45 ^{0}C was sufficient to enhance delivery when combined with GET. Administering GET to tissue with elevated temperature resulted in higher expression compared to delivery with GET alone (ambient temperature). In addition, expression could be obtained in

the deep dermis and muscle utilizing a surface electrode. This deeper penetration was not consistently obtained when GET was used without a thermal component. Interestingly, we have found that by elevating the temperature we could essentially reduce the applied voltage in half and still maintain the same level of expression. Observations have also revealed that successful delivery could be obtained at applied voltages that would not result in expression without the thermal elevation. Based on this observation, current work is evaluating approaches to achieve targeted delivery within the skin by selectively applying the heat and utilizing applied voltages that will only deliver plasmid DNA in combination with increased temperature.

43. A High-Throughput Screening Platform to Identify Nanoparticle Formulations for Transfection of Primary and Post-Mitotic Differentiated Cells

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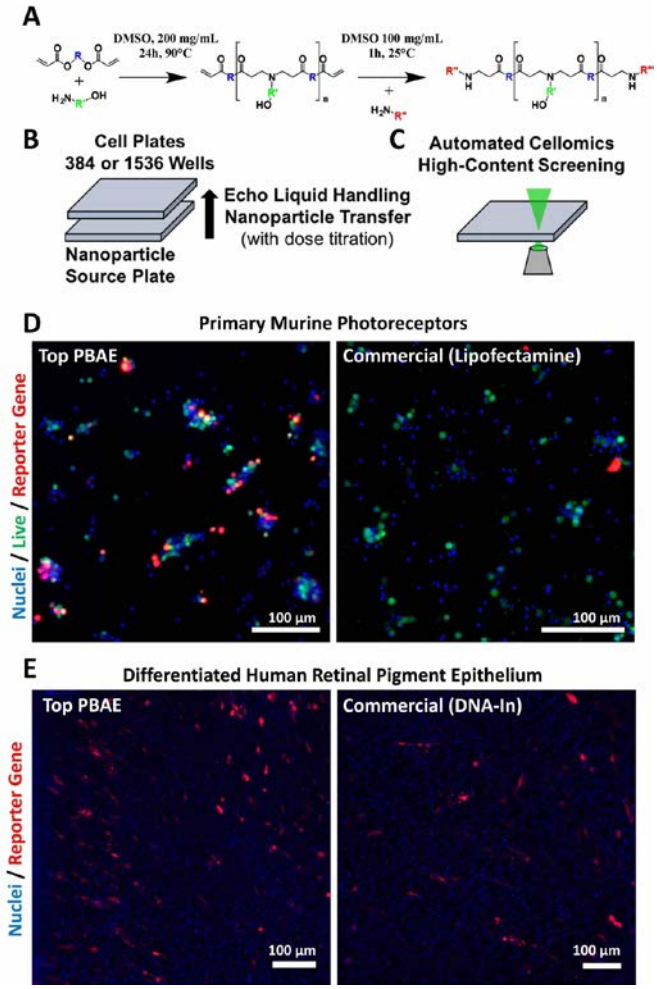

Purpose: Primary or differentiated, post-mitotic cells are often preferred tools over transformed or immortalized cell lines as they are biologically more relevant with expression profiles that more closely match those observed *in vivo*. Efficient gene transfer into post-mitotic cells would facilitate analysis of such cells *in vitro* and could also aid in the transfer of laboratory discoveries to the clinic. While viral strategies of DNA transduction can be effective at delivering nucleic acids to primary post-mitotic cells, efficiency of transduction and associated cellular toxicity can be very cell type-specific. To address these issues, we have developed biodegradable polymeric nanoparticles for efficient and safe transfection of hard to transfect primary or differentiated cells. These nanoparticles can deliver genes in vitro to primary photoreceptor (PhR) cells and differentiated retinal pigmented epithelial (RPE) cells with relatively low toxicity and transfection efficiencies of up to 30% in complete media, significantly higher than tested commercial reagents.

Methods: Dissociated primary murine PhR and human embryonic stem cells differentiated to post-mitotic RPE cells were seeded on 384-well or 1536-well tissue culture plates approximately 24h prior to transfection. A library of >100 linear and branched poly(beta-amino ester) (PBAE) polymers were synthesized from small molecule acrylate and primary amine monomers (Fig A), after which self-assembly of cationic polymer with plasmid DNA in buffer formed the polyplex nanoparticles in a multi-well nanoparticle source plate. Nanoparticles were then dispensed using an Echo 550 Series Liquid Handler (Labcyte) at DNA doses between 12.5-200 ng/well (Fig B) and incubated with cells in complete medium for two hours before replacement with fresh media. Approximately 48h post-transfection, cells were stained and images were acquired using an automated fluorescence-based imaging system (Cellomics ArrayScan VTI) (Fig C) to assess transfection efficacy and cytotoxicity of PBAE nanoparticles compared to the commercial transfection reagents, Lipofectamine 2000/3000, DNA-IN and JetPEI.

Results: A subset of polymeric PBAE nanoparticle formulations were identified that showed enhanced transfection compared to commercial reagents with cell type specificity determined primarily by the polymer structure endcap monomer. For primary PhR cells, PBAEs achieved >25% transfection efficacy, compared to <5% for tested commercial reagents (Fig D). Similarly, for differentiated human stem cell-derived RPE cells, PBAEs transfected up to nearly 30% of cells compared to <15% for tested commercial reagents (Fig E).

Conclusions: We have established a high-throughput platform to screen nanoparticles created from a wide variety of polymers for their ability to transfect retinal cells. Using this system, we have identified synthetic polymers that can be used for high efficacy non-viral gene delivery to primary murine PhRs and human stem cell-derived RPE cells, enabling cell signaling and developmental pathways to be more thoroughly studied at the molecular level. This platform can be used to identify the optimum polymer, w/w ratio of polymer to DNA and dose of nanoparticle for different retinal cell types among other post-mitotic cells.

44. A Systematic Evaluation of Factors Affecting Extracellular Vesicle Uptake and Functional Cargo Delivery

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Extracellular vesicles (EVs) are nanoscale lipid particles secreted by nearly all cell types. Recently, EVs have been identified as mediators of intercellular communication in both normal and disease processes through the transfer of encapsulated proteins and RNAs to recipient cells. This property makes EVs attractive vehicles for the delivery of therapeutics to specific cells. However, many aspects of EV function have yet to be elucidated. Specifically, the roles of factors such as vesicle subpopulation, producer and recipient cell pairing, and the display of receptor-targeting peptides on influencing particular uptake pathways and thereby functional delivery have not been fully evaluated. We investigated these factors using the breast cancer cell lines MCF-7 and MDA-MB-231 as model platforms. We discovered that cells uptake EV subpopulations isolated by different methods to different degrees, leading to functional cargo delivery in cases where membrane fusion with recipient cells occurs. Such membrane fusion allows for direct transfer of membrane proteins and release of EV contents into the cytoplasm of recipient cells, avoiding cargo degradation associated with the endocytic pathway. In contrast, the nature of the producer cells did not influence EV uptake, as recipient cells did not preferentially uptake vesicles that originate from their own cell type versus other cell types. One of the intriguing aspects of using EVs as drug delivery vehicles is the potential to deliver cargo to specific cells by expressing targeting peptides on the EV surface. By comparatively evaluating a series of targeting peptides reported to target breast cancer cells, we observed that display of such targeting peptides on the surface of EVs can increase, decrease, or have no effect on uptake; this range of impacts may reflect differences in receptor trafficking in the recipient cells. Altogether, these findings inform the design of EV-based therapies to confer efficient, targeted delivery of cargo to recipient cells.

45. Mucus-Penetrating Non-Viral Gene Delivery Platform for Obstructive Lung Diseases

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Introduction: One of the hallmarks of obstructive lung diseases is mucus accumulation in lung airways, which leads to series of pathological events including impaired mucociliary clearance, chronic infection and inflammation as well as airway obstruction. Gene therapy has emerged as an attractive approach due to the identification of novel genetic targets for curing obstructive lung diseases. However, inhaled gene therapy has failed to show clinical benefits to date at least partially due to inability of inhaled gene vectors to overcome viscoelastic mucus gel layer lining the airway epithelium. We developed biodegradable DNA-loaded mucus-penetrating DNA nanoparticles (DNA-MPP) that are minimally adhesive to mucus and thus capable of reaching the airway epithelium following inhalation. We have previously confirmed that DNA-MPP rapidly diffuse through sputum spontaneously expectorated from cystic fibrosis (CF) patients and mediate efficient transgene expression to the lungs of healthy mice. In this study, we have evaluated the performance of our system in a preclinical setting closely mimicking CF lung phenotype. **Methods**: Airway distribution and *in vivo* gene transfer efficacy of DNA-MPP were assessed using a transgenic mouse model characterized by airway mucus accumulation and chronic inflammation (i.e. Scnn1b-Tg mice), following an intratracheal administration via a Penn-Century microsprayer. Distribution of DNA-MPP and control gene vectors incapable of penetrating airway mucus (i.e. DNA-loaded conventional particles or DNA-CP) in lung airways was assessed by examining lung sections from treated Scnn1b-Tg mice using confocal microscopy, followed by an image-based, blinded analysis. Whole lung homogenate-based luciferase assay was conducted for the measurement of overall reporter transgene expression levels following inhalation of either DNA-MPP or DNA-CP. **Results**: DNA-MPP were found uniformly distributed throughout the airways of "mucus-hypersecreting" Scnn1b-Tg mice following inhalation, whereas DNA-CP were sparsely distributed as aggregates most likely due to their inability to efficiently penetrate airway mucus. Consequently, reporter transgene expression achieved by DNA-MPP was markedly greater compared to DNA-CP in the lungs of Scnn1b-Tg mice. **Conclusion**: We confirmed that DNA-MPP, but not DNA-CP, were able to overcome the airway mucus barrier and reach the underlying epithelium *in vivo*, thereby leading to efficient reporter transgene expression in the lungs characterized by CF-like lung disease. Follow-up studies include assessment of transgene expression kinetics, repeated dosing as well as therapeutic efficacy following administration of DNA-MPP carrying disease-curing genes. We acknowledge the financial support of NIH R01HL127413 and CFF HANES15G0.

46. New Recombinant Fusion Gene for Treatment of Obesity and Metabolic Disorders

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Obesity and its related metabolic comorbidities, including diabetes and fatty liver disease, represent a growing public health problem. Here we developed a rationally designed therapeutic gene named *EAT* to concurrently target excess energy intake and chronic inflammation, two major components of obesity pathophysiology, as a new strategy to treat this cluster of diseases. The dual functionality of EAT was accomplished by bridging exendin-4, a potent appetite-suppressing peptide, with human alpha-1 antitrypsin (AAT), a natural anti-inflammatory protein, through a short peptide linker consisting of tandem glycine/ serine residues. When delivered *via* a standard hydrodynamics-based procedure with dose of 20 microgram plasmid DNA per mouse, *EAT* gene transfer maintained EAT protein levels at 10^6 ng/ml in blood for over 3 weeks. Three repeated injections of EAT plasmid DNA, with intervals of 21 days, generated consistent patterns of transgene overexpression, indicating no stimulation of immune response. *Sleeping*

Beauty Transposon-based Tet-On/Tet-Off system was explored to achieve regulated EAT expression. Data show that *EAT* expression can be precisely controlled by administration of doxycycline inducer in both mice and cultured hepatocytes. Single injection of *EAT* gene induced 25% weight loss of high fat diet-induced obese C57BL/6 mice, reversed fatty liver and restored glucose tolerance within 3 weeks. The metabolic improvements are correlated with repressed energy intake and relieved adipose inflammation. In *ob/ob* mice, *EAT* gene transfer suppressed weight gain, blocked hepatic fat deposition, and maintained insulin sensitivity. Similar metabolic benefits was also achieved by repeated administration of EAT recombinant protein. At the molecular level, *EAT* gene transfer down-regulates transcription of pivotal genes responsible for lipogenesis (*Srebp-1c*, *Acc1*, *Fas*, and *Scd*) and lipid droplet formation (*Ppar*gamma, *Cd36*, *Fabp4*, and *Mgat1*) in the liver and chronic inflammation (*F4/80*, *Mcp1*, *Tnf*alpha, and *Il6*) in visceral fat, while promoting thermogenic gene expression (*Ucp1*, *Pgc1*alpha, *Dio2*, and *Elovl3*) in brown adipose tissue. These results suggest that we have created a new and fusion gene *EAT* for restoring metabolic homeostasis and nonalcoholic fatty liver disease.

47. *Clostridium* as "Trojan Horse" Vectors for Cancer Treatment

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Although cancer therapies and survival rates have substantially improved in recent years, the main issues of current treatments remain toxicity as well as the limited uptake within tumours. Overcoming the limited penetration of therapeutic agents into tumours without added toxicity is a challenging problem for which the use of bacteria can provide an ideal solution. *Clostridium sporogenes* is well-suited for this purpose being a non-pathogenic, spore-forming and proteolytic anaerobe; upon injection, spores will germinate and thrive exclusively in necrotic tumour regions, resulting in cancer-specific colonisation. Tumour necrosis is a histological feature of solid cancers and a marker of poor prognosis. Despite the high frequency of tumour necrosis, no treatment modalities exist to explicitly address this clinical issue and achieve a therapeutic index. In our approach, we exploit presence of necrosis to precisely target recombinant clostridia to tumours, where they can act as Trojan horse vectors, expressing and producing therapeutic agents at the site of action. *C. sporogenes* NCIMB10696 has been classified by the ATCC as a harmless biosafety level 1 organism. We determined its full genome sequence (GenBank CP009225). The assembly and annotation did not reveal the presence of any induced prophages or plasmids. Likewise, BLAST and PCR analysis confirmed the absence of toxin-encoding genes or remnants. In addition, the sensitivity of *C. sporogenes* towards a wide range of different antibiotics was established experimentally. Bioengineering of the *C. sporogenes* chassis has progressed to an unprecedented level of sophistication due to the technological development of tools and techniques. We have autonomous cloning, expression and knock-in vectors for the engineering of its genome and have developed patented technology that allows rapid and stable insertion of DNA into the *C. sporogenes* genome without antibiotic resistance markers. The generation of a research-derived codon optimisation algorithm as well as the creation of an extensive promoter and ribosome binding site library allowed us to identify strong signals for gene expression. We screened a phylogenetically diverse library of 85 nitroreductase (NTR) candidate genes from 19 bacterial species representing 13 different enzyme families. Using bacterial DNA damage and drug metabolism screens, we determined the NfsA and NfsB NTR families as being of particular interest. We also developed a series of mustard-based prodrugs that are excellent substrates for these NTRs, being readily reduced to cytotoxic metabolites able to diffuse from necrotic regions into surrounding viable tumour tissue. *In vivo* efficacy data confirmed the validity and proof-of-principle of our approach. In addition to prodrug activation, we have also shown that NTRs can metabolise clinical stage 2-nitroimidazole (2-NI) PET probes, widely used for detection of tumour hypoxia. Encouragingly, significantly higher tumour-to-blood ratios were observed for the NTR+ve tumours as compared to the parental xenografts. The preclinical evaluation of PET-guided NTR-recombinant *C. sporogenes* in combination with clinically tolerable prodrugs is an important step to the translation of this approach towards clinical applications.

48. Vector-Free Genome Editing of Primary Immune Cells for Cell Therapy

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While the *ex vivo* manipulation of primary cells has signaled a new era in the application of cell-based therapies, common methods to manipulate primary cells have limitations. To overcome the limitations associated with conventional cell delivery for engineering cell function, we have developed an approach where cells are passed through a constriction that results in temporary membrane disruption (CellSqueeze Technology). While the membrane is disrupted, material from the surrounding buffer can diffuse directly into the cytosol. This system has demonstrated efficacy in patient-derived cells, such as stem cells and immune cells, with a variety of molecules that are difficult to address with alternative methods. Moreover, by eliminating the need for electrical fields or exogenous materials such as viral vectors and plasmids, it minimizes the potential for cell toxicity and off-target effects.

Here, we present evidence detailing our ability to deliver functional material to primary human T cells and CD34+ cells with little detectable perturbation in baseline gene expression and cell function. We designed a series of experiments to manipulate gene expression with the CRISPR-CAS9 system using CellSqueeze to deliver CAS9 ribonucleoproteins (RNPs; recombinant CAS9 protein complexed with a single-guide RNA) designed to edit model loci CCR5 and B2M. We show that the delivery of the CRISPR-CAS9 system via the CellSqueeze technology results in significant CCR5 and B2M mutagenesis. To determine effect of CellSqueeze on gene expression and to compare to other delivery

systems, human CD34+ cells and T cells (n = 3 donors) were subjected to CellSqueeze or electroporation and gene expression changes were compared to unmanipulated control cells using microarray analysis. Differential gene expression with respect to both methods of delivery was assessed by performing t tests on the coefficient of a linear mixed-effects model that treated delivery method as a fixed effect and donor as a random effect. Electroporation produced substantially more changes in gene expression than CellSqueeze as compared to untreated controls in both cell types.

The functionality of T cells after CellSqueeze and electroporation were determined using a competitive homing mouse assay. T cells from CD45.1 mice were subjected to CellSqueeze while T cells from CD90.1 mice were electroporated, the cells were mixed at a 1:1 ratio, and injected into mice (2M cells/mouse). After 1 day the blood, spleen, and lymph nodes were harvested and FACS analysis was performed on recovered T cells. Despite being injected at a 1:1 ratio, over 80% of the T cells recovered from the target homing organs had been treated with CellSqueeze as opposed to electroporated, indicating T cells more effectively home to tissues after CellSqueeze. To assess CD34+ cell function, cells were plated in a Colony-Forming Cell (CFC) assay immediately post-delivery or injected into a NSG mouse model after an overnight culture. Cells subjected to CellSqueeze proliferate, differentiate, and engraft at rates similar to unmanipulated control cells. These data suggest that CellSqueeze is a viable delivery method for genetic engineering of primary human CD34+ cells with little impact on baseline gene expression or the ability of hematopoietic progenitors to proliferate and differentiate. The ability to deliver structurally diverse materials to difficult-to-transfect primary T cells and CD34+ cells indicate that this method could potentially enable many novel clinical applications.

Immunotherapy Clinicial Trials for Cancer

49. EBV-Specific T Cells Outside of the HSCT Setting

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Given the efficacy of donor-derived EBV-specific T cells (EBVSTs) in the post- HSCT environment, and considering many lymphoma patients are immunocompromised, we hypothesized that third-party EBVSTs can effectively treat EBV+ lymphoma by directly killing lymphoma cells, but more importantly, reactivating endogenous immunity. To test this, we designed a Phase 1 dose escalation trial of administration of third party EBVSTs to patients with relapsed/refractory EBV+ lymphoma. First, we generated a third-party bank from eligible donors based on racial diversity in order to represent diverse HLA haplotypes. Donors were screened with IFN-γ Elispot assays using overlapping peptide libraries (pepmixes) spanning the EBV Type 2 latency antigens (LMP1/2, EBNA1, BARF1), then selected based on antigen specificity. To characterize HLA restriction, we used peptides or pepmix-pulsed lymphoblastoid cell lines (LCLs), HLA matched at a single class I or II allele as targets in cytotoxicity assays. We then constructed a database containing the HLA restriction of antigen specificity for each line. This strategy ensures we only choose lines with antigen-specific activity restricted by alleles shared between donor and recipient. Our third-party EBVSTs exhibited significant specificity (75% of donors recognized ≥ 2 antigens) and cytotoxicity to pepmix-pulsed HLA-matched EBV-LCLs, with no activity against mismatched LCLs. The EBVSTs are polyclonal with a predominantly central memory phenotype, and polyfunctional, expressing IFN-γ and CD107a after coculture with HLA-matched LCLs. We have received 32 screening referrals, and have identified a suitable partially matched EBVST line with antigen-specific activity through shared alleles for 30/32 patients (94%). The average timeframe from referral to identification of suitable EBVST line was 1 week, substantially faster than with donor-derived EBVSTs due to the lengthy manufacturing process. We have treated and performed post-infusion evaluations on 7 patients. We observed enhanced and sustained viral and non-viral tumor antigen recognition (peaking at 2-4 weeks post-infusion), with continued antigen recognition up to 9 months in 2 responding patients, and loss of antigen recognition by 6 weeks in one patient with progressive disease. This epitope spreading is encouraging, since it implies that third-party T-cells may kick-start the patient's own antitumor immune response, a response that should be maintained long-term. 4/7 patients infused to date have had clinical benefit: 2 complete responses, 1 partial response, and 1 with stable disease without additional therapy at 12 months post infusion. 2 patients have received multiple infusions due to favorable clinical responses. The main challenge for this approach remains ensuring our allogeneic EBVSTs are not rejected by the host before they can eliminate lymphoma. We are therefore exploring adding lymphodepletion with hopes of enhancing persistence and antitumor activity.

50. Long-Term Follow-Up Data from 126 Patients with Recurrent High Grade Glioma from Three Phase 1 Trials of Toca 511 and Toca FC: Update and Justification for a Phase 2/3 Trial

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Toca 511 (vocimagene amiretrorepvec) is an investigational, conditionally lytic, retroviral replicating vector (RRV). The vector selectively infects cancer cells in animals and man, because productive infection is dependent on cell division and viral replication is favored in tumors by the immune suppressed microenvironment and by cancer-specific defects in innate immunity. Toca 511 spreads through cancer cells and stably delivers the gene for an optimized yeast cytosine deaminase that, upon administration of the prodrug Toca FC (an investigational, extended-release version of 5-fluorocytosine) generates 5-fluorouracil (5-FU) only in infected tissues (i.e. the tumor). The combined treatment is designed to directly kill cancer cells leading to limited inflammation and activation of antigen presenting cells. 5-FU can also kill nearby immune suppressive myeloid cells, while leaving systemic immunity intact, leading to enhanced anti-tumor activity. The safety, viral kinetics, immune response, and preliminary efficacy of this combined treatment have been investigated clinically since 2010 in three Phase 1 studies of 126 treated patients with recurrent high grade glioma (rHGG), each evaluating different methods of Toca 511 administration. Repeated courses of oral Toca FC followed Toca 511 administration. Results to date include good tolerability; no persistent viremia; successful gene transduction within resected tumors: no evidence for clonality of infected cells; evidence for tumor selective infection in humans; and increased median overall survival compared to historical controls with all three methods of vector administration. In addition, complete and partial responses with a median duration of initial response of > 25 months occur in patients starting 6-19 months after Toca 511 administration are associated with a long term survival; we also observed changes in immune cell populations, including increases in activated CD 4 and CD 8 T cells, in blood during treatment. These observations support an immune mechanism of action. Examination of IDH1 mutation status shows patients with a response are either wildtype or mutant and with enrichment for CR in IDH1 mutants with first recurrence. Neo-antigen load in available pretreatment tumor samples is being evaluated. Preliminary data from these studies supported conduct of a randomized, Phase 2/3 study in patients with rHGG (NCT02414165) in 2015. Updated data from the Phase 1 studies will be presented.

51. Induction of APOBEC Expression During Multiple Types of Immuno, Gene and Viral Therapies Enhances Tumor Cell Escape

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Tumor escape from frontline therapy is a major clinical problem. We have used three different treatment models to recapitulate this phenomenon in animal models using treatment of established subcutaneous tumors with either: adoptive T cell therapy, suicide immuno-gene therapy or oncolytic viroimmunotherapy. During our experiments using OT-I adoptive T cell therapy to treat B16ova tumors, we observed that about 50% of mice in which primary tumors initially regressed completely underwent tumor recurrence at later time points. In some cases the recurrent tumors had lost the gene for OVA against which the OT-I T cells were initially targeted. However, a proportion of recurrent tumors retained both the ova gene and expressed ova mRNA. Of these cases, 4 of 5 tumors contained a TCA to TTA conversion within the *ova* gene which created a STOP codon upstream of the SIINFEKL epitope recognized by the OT-I T cells. TCA to TTA is characteristic of mutations induced by members of the APOBEC family of cytidine deaminases expressed by normal cells as a means to restrict viral infection. We confirmed that APOBEC3 is induced strongly in tumor cells undergoing T cell attack, suicide gene therapy and oncolytic viral therapy. Moreover, recurrent B16tk tumors recovered from mice which had initially undergone complete macroscopic regression following Ganciclovir chemotherapy contained a similar signature of APOBEC-mediated mutation to introduce STOP codons within the HSVtk gene. Finally, we have shown that APOBEC3 expression is induced by infection with both VSV and Reovirus oncolytic viruses and that emergence of virus resistant cells can be significantly inhibited using shRNA to target APOBEC3 expression. Conversely, overexpressing APOBEC3B increases the number of clones that were able to escape oncolysis. The tumor cells which survived *in vitro* after prolonged culture with virus were themselves significantly more resistant to subsequent infection/oncolysis with the parental virus if they were forced to express APOBEC3B during viral infection. These data suggested that APOBEC expression leads to cellular mutations which select for virus resistant tumor cells, a hypothesis which is supported by the finding of multiple mutations in matched tumor cell lines either over-expressing APOBEC3B or not. In addition, virus stocks recovered from long term passage through B16 cells over-expressing APOBEC3B had significantly lower titers than the parental virus stock and were less oncolytic when used to infect parental tumor cells. These data indicate that APOBEC induction by oncolytic virus infection also contributes to the emergence of virus resistant tumor cells by decreasing the fitness of the oncolytic agent.

Taken together, these data show that targeting APOBEC expression during multiple forms of immune, gene and, viral therapy may decrease the ability of tumor cells to escape the frontline therapeutic pressure.

52. 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, Adeno associated vectors (AAV) 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. VDJ targeting may have several advantages over state of the art technologies: Only developing lymphocytes, expressing RAG, will incorporate the receptor/Ab gene, which will thus be expressed in potent naïve cells. Targeted T cells will express only the desired receptors, due to allelic exclusion. Targeted B cells will express the transgene as a B cell receptor, and upon activation will 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*. We will present early proof of concept experiments in immortalized and inducibly differentiating lymphocytes.

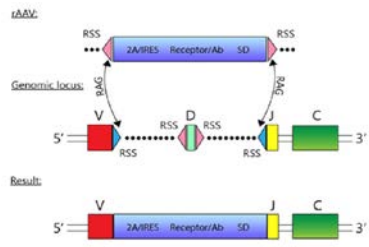

Figure 1: VDJ targeting: The rAAV vector encodes a receptor or Ab gene flanked by RSSs. Transduction of lymphocyte precursors, which express RAG, facilitates insertion of the receptor/Ab gene between gene segments. In the example presented, the transgene cassette is targeted between a V segment and a 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

53. In Vivo Gene Transfer and Hepatocyte-Restricted Transgene Expression in a Murine Model of On-Target Off-Tumor Cytotoxicity by CAR T Cell Therapy

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Chimeric antigen receptor T cells (CARTs) can target malignant cells by recognizing tumor-associated antigens (TAA) but autoimmune toxicity can occur if TAAs are expressed on normal cells even at low levels. We developed a mouse model, in which human TAA transgenes are expressed at varying levels in the liver and can be used as targets for CAR T cells. We chose human Her2 as a TAA, and injected mice IV with AAV8-Her2 at varying titers, and observed the expected dose-dependent Her2 expression in murine hepatocytes. This model was used to compare toxicity between T cells that expressed either high- or low-affinity Her2 CARs. In mice expressing high levels of hepatic Her2 ($Her2^{high}$), both the high- and low-affinity CARTs caused severe liver toxicity as indicated by a four-fold increase in serum ALT levels and uniform mortality relative to Her2-negative controls (100% vs 0% 22 days post-CART infusion). In mice expressing low levels of hepatic Her2 ($Her2^{low}$), the high-affinity CARTs caused significantly more liver toxicity than the low-affinity CARTs, suggesting that low-affinity CARTs were better tolerated. Next, we compared the anti-tumor effects of high- and low-affinity CARTs in mice with both $Her2^{high}$ tumor xenografts and $Her2^{low}$ livers, as a model for relative TAA expression in patient tumors and normal tissues. *In vivo* imaging revealed that high-affinity CARTs exhibited longer retention time in the liver compared to low affinity CARTs and to negative controls (12, 8 and 4 days, respectively). Consequently, the low-affinity CARTs were observed infiltrating the tumor earlier than the high-affinity CARTs (8 vs 12 days) and the low affinity CARTs cleared the tumor more effectively. Overall, our model clearly shows a higher therapeutic index for the low-affinity CAR T cells versus high-affinity CAR T cells.

54. Disease Burden and Transplant on Long-Term Survival After CD19 CAR T Cells in Adults with Relapsed Acute Lymphoblastic Leukemia

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CD19-specific chimeric antigen receptor (CAR) T cells have demonstrated high initial responses in patients with relapsed B-cell acute lymphoblastic leukemia (B-ALL). However, clinical characteristics associated with the durability of response remain undefined. Herein, we report the results from analysis of our phase I clinical trial of 19-28z CAR T cells in adult patients with relapsed B-ALL with a focus on impact of pre-treatment disease burden and post-CAR T cell allogeneic transplant on long-term clinical outcome. Adults with relapsed B-ALL were infused with autologous T cells expressing the 19-28z CAR. Disease burden was assessed by bone marrow biopsy immediately prior to T cell infusion. We grouped patients into 2 cohorts based on disease burden upon T cell infusion: minimal residual disease (MRD) with <5% blasts in bone marrow and morphologic disease (≥5% blasts). Median follow-up duration was 18 months (range, 0.2-57.3). 51 adults received 19-28z CAR T cells; 20 in the MRD and 31 in the morphologic cohort. Complete remission (CR) rates were comparable (95% and 77%, respectively). However, median event-free and overall survivals widely diverged among the 42 patients who achieved MRD-negative CR: not reached (NR) (95% confidence interval [CI]: 4.2-NR) vs. 6.3 months (95% CI, 4.8-9.0) (p=0.0005), and NR (95% CI, 15.3-NR) vs. 17 months (95% CI, 8.5 - 36.2) (p=0.0189), in the MRD and morphologic cohorts, respectively. Subsequent allogeneic HSCT in either cohort did not improve survival (p=0.8). MRD cohort patients developed substantially less severe cytokine release syndrome (CRS) and neurotoxicity, both correlating with peak CAR T cell expansion (p=0.0326 and p=0.0001, respectively). Despite comparable initial CR rates between the two cohorts, durability of 19-28z CAR T cell mediated remissions and survival in adult patients with relapsed B-ALL positively correlated to a low disease burden and do not appear to be enhanced by allogeneic transplant. Our findings strongly support the early incorporation of CD19 CAR therapy before morphologic relapse in B-ALL.

55. CARs in Leukemia

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T cells bearing a second-generation anti-CD19 chimeric antigen receptor (CAR) induce complete remission in >90% of patients with acute lymphoblastic leukemia (ALL) at our institution. However, disease may recur and we recently identified two molecular mechanisms of relapse (PMID: 26516065). We here present a novel mechanism of antigen-negative relapse in a pediatric ALL patient. A 21 year-old male patient was in third relapse at the time of enrollment onto our CTL019 trial (ClinicalTrials.Gov # NCT01626495). The patient achieved an MRD-negative complete remission 1 month after CTL019 infusion but relapsed nine months later. Quantitative PCR analysis of the transgene and flow cytometry for CAR19 protein analysis showed the expected expansion of the CART cells followed by log-normal decay following disease eradication. At relapse, however, the CAR protein was found to be expressed by the now CD19-negative $CD45^{dim}CD10^{+}CD3^{neg}CD22^{+}$ leukemia and not T cells. Immunoglobulin heavy chain (IgH) gene rearrangement sequencing (NGIS) of the apheresis product, used for CTL019 manufacturing, and relapse marrow at 9 months demonstrated clonal identity of the relapsed clone, which carried two rearranged IgH alleles. Sequencing of the CD19, CD21, CD81, and CD225 loci did not reveal any mutations. The analysis of lentiviral vector integration sites (LVIS) of the infusion product and post-infusion specimens showed two LVIS, one located on > 50 kb distal from neuropilin (NRP1) and the other on a intron of proprionyl coenzyme A carboxylase-A (PCCA) but further flow cytometric and qRT-PCR analysis indicated that neither expression of NRP1 nor PCCA was affected. Investigation into the origins of the leukemic CAR transduction event showed that the patient did not exhibit replication-competent lentivirus. However, NGIS analysis of infusion product revealed the leukemic clonotypes, indicating that the gene transfer occurred during the manufacturing of the CTL019 cells. Our investigation into the biology of CAR19-expressing ALL cells showed the following: 1) the *in vitro* analysis of BBζ-signaling CAR19 showed no evidence of cytokine secretion; 2) the infusion of the baseline leukemia and CAR19-expressing leukemic cells from the same patient in mice did not demonstrate differential pharmacodynamics, even after restimulation with human CD19-expressing murine B cells *in vivo*; 3) the CD19 protein was detectable using flow cytometry and confocal microscopy, but only with an antibody recognizing an intracellular epitope; 4) the relapsed clone was indeed resistant to killing by CART19 cells in a xenograft model yet retained sensitivity to anti-CD22 CAR T cells; 5) we recreated in vitro and expanded $CAR19^{+}$ ALL cells in other patients, and are currently working on assessing the phenotype and function. In conclusion, our data therefore show that a single leukemic cell accidentally transduced with CAR19 survived the 10-day manufacturing process and, upon reinfusion into the patient, was the sole clone at relapse 9 months later. This leukemic clone evaded CTL019 detection via downregulation of the target antigen in a cell-autonomous fashion.

Cardiovascular and Pulmonary Gene and Cell Therapies

56. hsa-miR-665 Prevents Cardiomyocyte Hypertrophy and Preserve Normal Cardiac Function After Pressure Overload

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The adult heart is capable of remodelling in response to different pathological stimuli; in most cases, a phase of compensated hypertrophy evolves into frank dysfunction and heart failure. To identify microRNAs able to prevent cardiac hypertrophy and preserve cardiac function, we performed a high-content microscopy, high-throughput functional screening for human miRNAs able to reduce neonatal cardiomyocyte (CM) cell size using a whole-genome miRNA library. The most effective anti-hypertrophic miRNAs was hsa-miR-665. In a model of transverse abdominal aortic constriction (TAC) in 8 weeks old CD1 mice (n=14 per group), AAV9-mediated delivery of miR-665 showed remarkable capacity to protect against pathological cardiac hypertrophy and preserve function over time. This effect was observed when the vectors were delivered either before (LVEF at 60 day after TAC: 51.3% ±5.8 in treated vs 34.82% ±0.77 in controls; $P<0.005$) or after hypertrophy onset (LVEF at 60 days after TAC: 57.5%±5.60 in treated vs 28.4%±15 in controls; $P<0.001$). Global mRNAs changes in hearts treated with miR-665 were evaluated by mRNA deep sequencing. All the 90 genes that were found to be expressed ≤2 fold over control were individually downregulated by specific siRNAs and tested for being direct miR-665 targets. This approach identified three sarcomeric proteins as direct mediators of miR-665 activity, namely Enah, Fhl1 and Xirp2, which are known to be involved in sarcomeric I-band mechanotransduction and myofibrillar remodelling. In conclusion, miR-665 represents an important tool to decipher the molecular mechanisms of hypertrophy and offer a potential lead for the development of new biotherapeutics.

57. CRISPR/Cas9-Mediated Introduction of the Sodium-Iodide Symporter Gene Enables Non-Invasive *In Vivo* Tracking of Rhesus iPSC-Derived Cells

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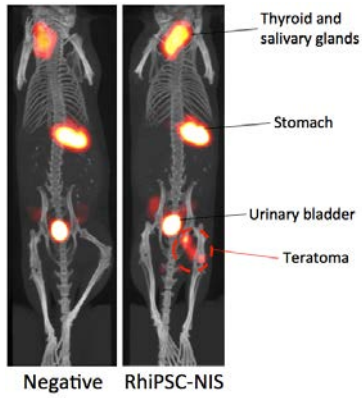
Due to their strong similarity to humans, non-human primate induced pluripotent stem cells (iPSCs) play an important role in investigating the safety and functional utility of iPSC-derived cells in preclinical models. However, there have been limited studies incorporating *in vivo* molecular imaging to provide long-term information regarding localization and persistence of iPSC-derived cells following engraftment. Here, we show a platform for using the sodium-iodide symporter (NIS), a non-immunogenic endogenous reporter gene, to enable non-invasive tracking of transplanted iPSC-derived cells via positron emission tomography-computed tomography (PET/CT). Endogenous NIS expression is largely confined to the thyroid, salivary glands, and stomach, and ectopic expression enables imaging with several clinically available radionuclide tracers. We recently demonstrated that the adeno-associated virus site 1 (AAVS1) could be used as a potential safe harbor locus for targeted integration of transgenes in rhesus macaque iPSCs (RhiPSCs). Using our optimized CRISPR/Cas9 mediated gene-editing protocol, we have successfully generated RhiPSC clones with rhesus NIS introduced at one or both alleles of the AAVS1 locus. The ten sites with highest probability for off-target activity in the rhesus genome were computationally identified, all of which were negative for indels in all tested clones. RhiPSC-NIS clones exhibited robust expression of NIS from a constitutive CAG promoter, confirmed by immunostaining. Using a standard radioisotope uptake assay, RhiPSCs-NIS showed significant uptake of the radiotracer compared to parental RhiPSC clones. Moreover, intracellular accumulation of the radiotracer was blocked by $KClO_4$, a specific inhibitor of NIS, indicating that uptake was NIS-dependent. To validate the functionality of the targeted NIS transgene *in vivo*, $5x10^6$ RhiPSCs-NIS were injected into the left hindlimb of immunodeficient mice. RhiPSCs-NIS-derived teratomas could be visualized by PET/CT as early as 2 weeks post-injection, prior to development of a palpable teratoma. Quantitative analysis of the PET/CT data over time showed that total intensity from the injection site as well as mean intensity steadily increased between 2, 4, and 6 weeks post-injection in all mice. Both monoallelic and biallelic RhiPSCs-NIS could be equally well detected. Figure 1 shows representative PET/CT images at 4 weeks post-injection. In conclusion, our data suggests that NIS-mediated *in vivo* imaging is feasible via safe-harbor targeting of NIS transgene in RhiPSCs. Directed differentiation of RhiPSCs-NIS to clinically relevant target cells, such as cardiomyocytes and hepatocytes, is ongoing and functional data will be presented.

Figure 1: *In vivo* imaging of RhiPSCs-NIS-derived teratoma. Representative PET/CT images of mice injected with parental RhiPSCs (left) or RhiPSCs-NIS (right) in the left hindlimb. The red oval highlights the signal from RhiPSCs-NIS-derived teratoma. Note the endogenous signals from the thyroid, salivary glands, and stomach, and that the bladder is also visible due to renal elimination of tracer.



58. Systemic Delivery of AAVB1-GAA Gene Therapy for Respiratory Pathology in Pompe Disease

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Pompe disease is a rare autosomal recessive disease due a deficiency of the lysosomal enzyme alpha-1-glucosidase (GAA). The lack of GAA results in systemic lysosomal accumulation of glycogen which causes destruction of skeletal muscle, CNS and smooth muscle. Respiratory insufficiency is a hallmark of Pompe deficiency, even in patients treated with enzyme replacement therapy. Classically, respiratory weakness has been attributed to skeletal muscle and motor neuron pathology. Here, we propose that 1) smooth muscle pathology resulting in weakness of the trachea and bronchi play an important role in Pompe disease and 2) that a newly engineered AAV, AAVB1, carrying the GAA gene can correct systemic and airway weakness in a Pompe disease mouse model (the $Gaa^{-/-}$ mouse). 3-month-old $Gaa^{-/-}$ animals were injected via tail vein with either AAVB1 or AAV9 vector containing the GAA gene expressed by the Desmin promoter. Additional $Gaa^{-/-}$ and wildtype animals were injected via the tail vein with PBS as controls. Using histological analysis and studies of pulmonary mechanics, we evaluated respiratory airway pathology in $Gaa^{-/-}$ animals compared to wildtype animals. PAS staining revealed large deposits of glycogen in the smooth muscle, cartilage and epithelium of the trachea and bronchi of $Gaa^{-/-}$ mice that was not observed in WT animals. Pulmonary mechanical studies revealed significantly hyporesponsive airways to increasing doses of methacholine implying abnormal airway constriction and caliber. In ongoing gene correction studies, we preformed behavioral testing and respiratory measures in awake spontaneously breathing animals. A significant increase in survival was observed in treated animals, with 7/8 (88%) animals reaching the experimental endpoint

for both AAV9 and AAVB1 groups, whereas only 4/9 (44%) *Gaa*$^{-/-}$ untreated animal survived until the experimental endpoint of 9-months of age. Animals were assayed at 4-months, 6-months and 9-months of age for in-life behavioral testing and whole body plethsmography to measure spontaneous breathing. Animals treated with AAVB1-GAA had an improvement in behavioral testing of 2-limb grip strength test and inverted screen particularly at 6-months of age. Preliminary data of breathing measurements in response to a respiratory hypercapnic challenge suggest that mice treated with AAVB1-GAA were able to maintain frequency of breath at near WT levels and there was a trend towards increased tidal volumes, and peak inspiratory flow, a measurement of diaphragm strength, at all time points compared to untreated *Gaa*$^{-/-}$ animals. In conclusion, these results suggest that lower airway smooth muscles of *Gaa*$^{-/-}$ animals have significant pathology resulting in hyporesponsive airways to bronchoconstrictor agonists and that a new AAV vector - AAVB1 - holds promise for correcting systemic and respiratory disease when administered to adult *Gaa*$^{-/-}$ animals.

59. Abstract Withdrawn

60. Pre-Clinical Development of AAV-Mediated *TAZ* Gene Delivery to Treat Barth Syndrome

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Recessive loss-of-function mutations in the gene encoding tafazzin (*TAZ*) are responsible for the development of Barth Syndrome (BTHS). Tafazzin is a nuclear-encoded protein that translocates to the inner mitochondrial membrane where it remodels monolysocardiolipin into cardiolipin. The maintenance of mature cardiolipin is essential for mitochondrial membrane stability and efficient respiratory chain function. In BTHS, this deficiency results in cardiomyopathy and skeletal muscle weakness. There are no effective BTHS therapies and treatments consist of symptom alleviation. Amongst available gene therapy vehicles, adeno-associated virus (AAV) stands out as a highly promising candidate for treating BTHS due to the safe, high level, and long-term expression it provides. To evaluate *TAZ* expression levels in a shRNA knockdown-based mouse model of BTHS following intravenous injections of 3 different AAV-*TAZ* vectors as compared to healthy and untreated BTHS mouse controls. BTHS mouse pups (1-3 days) and adults (3 months) were intravenously administered $1x10^{13}$ vector genomes/kg of dsAAV9-Des-*TAZ*, dsAAV9-CMV-*TAZ*, or dsAAV9-Taz-*TAZ* (8/group). Treated, untreated, and healthy control mice are evaluated by ECHO analysis at 3 and 5 months of age pre and post exposure to hypoxia to induce cardiac stress. Activity levels and exhaustion are measured in treated cohorts, untreated BTHS-mice, and healthy controls using the ActiTrack system. Tissues are harvested at 5 months of age and *ex vivo* force mechanics performed on solei muscles to further evaluate strength and fatigue *in situ*. Oxygen consumption assessments are performed on mitochondria from heart and muscle samples using the Oxytherm system following sequential addition of: glutamate, malate, ADP, oligomycin and CCCP. Mitochondrial morphology is evaluated by electron microscopy image analysis. *TAZ* expression levels are determined by RT-PCR and protein levels by western blotting. Data acquired from these ongoing studies show that BTHS mice from all treatment groups display improvements in multiple parameters. ECHO analyses of left ventricular wall dimensions *(IVSd, LVIDd and LVPWd)* showed normalization in all treatment groups as compared to controls both pre and post hypoxia. Heart weight/body weight ratios were also normalized in treatment groups with mean ratios ranging from 4.5 - 5.0 as compared to untreated BTHS mice with a mean ratio of 5.65 ± 0.12. ActiTrack-based movement analyses revealed no significant difference in total distance, vertical movement, resting time, fast movements, or fatigability in treatment groups as compared to healthy controls. Force mechanics data showed normalized solei strength and fatigability and oxygen consumption evaluations of isolated heart mitochondria suggest that treatment with any of the AAV vectors improves state 3, 4, and 5 respirations significantly above that of untreated BTHS mice to healthy levels. Electron microscopy analyses revealed improved mitochondrial cristae organization, mitochondrial contour ratios, and increased sarcomeric organization in treated groups. 1-5 fold increases in *TAZ* gene transcripts and tafazzin protein expression were also confirmed in all treatment groups. Our promising preliminary data suggest that gene therapy is an effective treatment for BTHS through prevention of the development of cardiac hypertrophy, maintenance of muscle strength, and improved mitochondrial structure and function. Successful completion of this study will provide preclinical data necessary for further translation of the most optimal vector system into the clinical realm.

61. Proof-of-Concept for Non-Nuclease-Mediated Genome Editing to Treat A-1 Antitrypsin Deficiency

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Alpha-one antitrypsin (AAT) deficiency is a common autosomal co-dominant genetic disorder. This condition affects 1:2500 individuals of European ancestry, leading to the development of lung and liver disease. Within North American and Northern European populations, an estimated 4% of individuals are carriers of mutant alleles, and 90% of affected individuals carry the Z mutation. AAT is a protease inhibitor predominantly synthesized in the liver that belongs to the serine protease inhibitor (serpin) family. Upon secretion into the blood stream, AAT enters the lungs where it inactivates excess neutrophil elastase, thereby preventing damage to the alveoli. Mutations of the *SERPINA1* gene can lead to reduced serum levels of AAT and decreased protein functionality, allowing for unrestricted elastin breakdown, pulmonary inflammation and eventual emphysema. Lung disease is the principal cause of death, however, AAT deficient subjects can

also suffer from liver disease of varying severity that stems from the accumulation of intrahepatic Z- AAT polymers. The current mouse model of the disease is a transgenic mouse expressing the mutant human Z-AAT gene in a C57BL/6 background (PiZ mouse). While this mouse does not develop lung disease due to the presence of murine AAT, intrahepatic accumulation of human Z-AAT does lead to liver disease. Previous experimental data showed that in these mice, liver-directed gene augmentation alone leads to toxicity. However, liver-directed gene augmentation with a previously developed dual function vector that simultaneously augments AAT and silences Z-AAT (1) is safe, suggesting that the Z-AAT liver burden needs to be alleviated if this organ is to be used for gene augmentation.We therefore developed an integrating promoterless AAV vector based on the dual function vector and the previously described GeneRide (2) approach. It is a promoterless cassette containing a 2A-peptide sequence followed by a de-targeted, c-Myc-tagged human AAT sequence, and an artificial miRNA targeting Z-AAT. The cassette is flanked by two homology arms that are complementary to the C57BL/6 *Alb* locus and allow homologous recombination (HR). Following HR, *Alb* and *AAT* will be co-transcribed as a single Alb-AAT mRNA and will lead to the production of two proteins through ribosomal skipping. Here, PiZ mice were treated with either saline or the integrating rAAV, and data shows that HR did occur in the liver of these animals. Moreover, the genome-corrected hepatocytes expand preferentially as compared to Z-AAT-burdened hepatocytes, leading to increasingly larger populations of genome-corrected cells over time. Overall, the presented data provides proof-of-concept for non-nuclease-mediated genome editing to treat α-1 antitrypsin deficiency and supports further development of this program. (1) Mueller, C. et al. Sustained miRNA-mediated knockdown of mutant AAT with simultaneous augmentation of wild-type AAT has minimal effect on global liver miRNA profiles. Mol.Ther. 20, 590-600, doi:mt2011292 [pii];10.1038/mt.2011.292 [doi] (2012).(2) Nygaard, S. et al. A universal system to select gene-modified hepatocytes in vivo. Sci Transl Med 8, 342ra379, doi:10.1126/scitranslmed.aad8166 (2016).

62. Development of NSC-Mediated Enzyme/ Prodrug Therapy for Small Cell Lung Cancer

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Small cell lung carcinoma (SCLC) is the most aggressive type of lung cancer and is closely associated with inhalation of tobacco smoke. SCLC metastases develop quickly, with the median survival in untreated patients of only 1-2 months from the onset of symptoms. Our long-term goal is to overcome the progression and recurrence of small cell lung carcinoma (SCLC), by developing novel tumor-specific anti-SCLC therapies. Many studies suggest the presence of a rare, drug-resistant population of cells (cancer stem cells; CSCs) in solid tumors and leukemia that possess the capability to regenerate and propagate the tumor, serving as the underlying cause of tumor recurrence. Current treatment strategies may fail to target this drug-resistant subpopulation, which may explain the initial therapeutic response of the majority of tumor cells that is followed by a later recurrence. We have previously demonstrated that neural stem cells (NSCs), engineered to secrete a modified human carboxylesterase (hCE1m6; hCE1m6-NSCs) can selectively localize to metastatic solid tumor foci in multiple organs following intravenous administration, and convert the prodrug CPT-11 (Irinotecan; IRN) to the 1000 fold more potent topoisomerase-1 inhibitor SN-38, resulting in significant therapeutic efficacy. In the current pre-clinical studies, we apply this NSC-mediated enzyme/prodrug gene therapy to SCLC. We have now determined the *in vitro* IC50 values of 2 human derived SCLC lines to SN-38, IRN only and IRN + hCE1m6-NSC conditioned media. IC50 values of IRN were decreased by 3000 to 4000-fold when IRN was used in combination with the hCE1m6-NSC conditioned media for all SCLC cell lines. We then investigated hCE1m6-NSC biodistribution and prodrug conversion in subcutaneous immunodeficient mouse models of human SCLC. In this proof-of-concept study, we intravenously administered hCE1m6-NSCs, followed by human equivalent doses of IRN. Immunohistochemistry (IHC) and pharmacokinetic (PK) analysis demonstrated biodistribution of NSCs and localized conversion of IRN to SN-38 in SCLC tumor models and demonstrated tumor localized conversion of IRN to SN-38. We postulate that this NSC mediated enzyme/prodrug gene therapy strategy would provide a more effective, tumor selective, and potentially less toxic treatment for SCLC patients.

Gene and Cell Therapies for Hematologic and Immunologic Diseases I

63. Efficacy and Safety of Liver-Directed Lentiviral Gene Therapy in Hemophilia B Dogs and Non-Human Primates

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Lentiviral vectors (LV) are attractive vehicles for liver-directed gene therapy by virtue of their ability to stably integrate in the genome of target cells and the low prevalence of pre-existing immunity against HIV in humans. Over the past years, we have developed a LV platform that can achieve stable transgene expression in the liver, induce transgene-specific immune tolerance and establish correction of hemophilia in animal models upon systemic administration. These LV are designed to stringently target transgene expression to hepatocytes through transcriptional and microRNA-mediated regulation. We have previously evaluated portal vein administration of LV expressing canine factor IX (FIX) in 3 adult hemophilia B dogs and reported stable multiyear reconstitution of FIX activity up to 1 % of normal. We have more recently treated 3 dogs as pups (2-4 months of age) by peripheral vein administration of escalating doses of LV expressing a hyper-functional canine FIX and achieved reconstitution of FIX activity

up to 32% of normal at the highest dose tested. Moreover, we have recently initiated a study in non-human primates (NHP) to evaluate the acute toxicity, transduction efficiency, human FIX expression and biodistribution of two versions of LV administered by a peripheral vein: one version has increased levels of CD47, a phagocytosis inhibitor, on the vector surface (CD47hi-LV), and showed decreased transduction of human macrophages *in vitro* and mouse spleen and liver macrophages *in vivo*. We have now administered LV or CD47hi-LV to 6 NHP (3 for each LV version) at $7.5x10^9$ transducing units/kg of body weight. We chosed *Macaca nemestrina* as host specie, because of the lack of relevant restrictions to LV transduction. LV administration was well tolerated, without significant elevation of serum aminotransferases or increase in body temperature and only caused a transient self-limiting leukopenia for 1-2 days after administration. Besides being normal from the coagulation stand-point, all LV-treated animals showed further sustained shortening of the clotting time after LV administration, suggesting an increase of functional FIX expression. Evaluation of human FIX expression in the plasma of treated animals is ongoing and will be reported, together with LV circulating half-life and the inflammatory cytokines response following LV administration. We will assess LV DNA biodistribution in the liver and major organs 3 months after treatment. This pilot NHP study will be crucial to inform further development of our LV-based gene therapy strategy for hemophilia and, if a favorable efficacy and safety profile will be confirmed, it will suggest that LV may complement other available vectors to address some of the outstanding challenges posed by liver gene therapy of hemophilia and conceivably other diseases.

64. CD20 Receptor Targeted Lentiviral Gene Transfer of IGG-Fusion Protein into B Cells to Induce Tolerance in Hemophilia B Mice

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Complications to factor IX (FIX) replacement therapy in hemophilia B may occur in the form of neutralizing inhibitory antibodies to the infused protein. Although the frequency of inhibitor occurrence is low, life threatening complications, such as allergic/anaphylactic reactions are frequently associated with inhibitory antibody (inhibitor) development. Current options for FIX inhibitor management are limited, which puts focus on alternative approaches for the prevention or treatment of inhibitors. Gene modified B cells expressing immunoglobulin G (IgG) fusion proteins have been shown to induce tolerance in several autoimmune and other disease models. We have previously shown that B cell based therapy for hemophilia B, using retroviral transduction of LPS activated B cells, effectively prevented inhibitor formation to FIX and desensitized mice with a pre-existing response. In this study, we developed a lentiviral vector (LV) delivery system for transducing human B cells. Envelope glycoproteins commonly employed by LV fail to transduce resting human B cells and lack cell specificity, which has been an obstacle for translation of this approach. We overcame this hurdle by engineering measles virus (MV) envelope glycoproteins to express a single-chain variable fragment (scFv) specific for human CD20 (hCD20), in order to re-target the vector to CD20 expressing human B cells. In addition to remarkable specificity, the re-targeted MV-LV system was able to transduce resting B cells with minimal production of inflammatory cytokines, making it uniquely suited for in vivo cell targeting. Transduction efficacies in either primary human CD20 expressing B cells or transgenic mice expressing hCD20-tg B cells on a BALB/c background were from 39-60%. Using this strategy, we could demonstrate induction of humoral tolerance, where adoptive transfer of hCD20-tg B cells transduced with MV-LV expressing an IgG-hFIX fusion construct resulted in suppression of antibody formation against FIX in adjuvant, delivered by the SC route in a mouse model of hemophilia B (BALB/c-HB mice). Prevention of inhibitor formation to hFIX administered via the more clinically relevant IV route (3 IU hFIX, 1x/ week for 8 weeks) was also observed, where 3 out of 4 mice receiving B cell gene transfer did not develop inhibitors against FIX, while ¼ animals (25%) formed an inhibitor. This was in contrast to the 81% inhibitor response rate seen in control animals that received protein therapy only. In conclusion, transduction of receptor-specific re-targeted LV into resting B cells is a promising method to develop B cell therapies for antigen-specific tolerance induction in human disease.

65. T Cell Gene Therapy Corrects Humoral and Cytotoxic Defects in X-Linked Lymphoproliferative Disease (XLP)

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X-linked lymphoproliferative disease (XLP) arises from mutations in the *SH2D1A* gene encoding SAP, an intracellular adaptor protein expressed in T, NK and NKT cells. SAP is a key regulator of immune function and deficiency causes abnormalities of NK cell cytotoxicity, NKT cell development and T cell dependent humoral function. The absence of SAP in $CD4^+$ T follicular helper (T_{FH}) cells leads to defective long-term humoral immunity. Clinical manifestations are characterised by haemophagocytic lymphohistiocytosis (HLH), lymphoma and dysgammaglobulinaemia. Curative treatment is limited to allogeneic haematopoietic stem cell transplant with outcome reliant on a good donor match. We have previously shown correction of cellular and humoral immune defects in a $SAP^{-/-}$ mouse model using lentiviral mediated gene correction in haematopoietic progenitors providing proof of concept for gene therapy as a potentially curative treatment. Given that the majority of symptoms arise from defective T cell function, we also investigated whether the infusion of gene corrected T cells could correct known effector cell defects associated with the condition. We initially confirmed that transfer of wild type T lymphocytes into $SAP^{-/-}$ mice improves humoral defects characterised in this model. Subsequently CD3+ lymphocytes from $SAP^{-/-}$ mice were transduced with a gammaretroviral vector containing codon optimised human SAP cDNA before infusion into sub-lethally irradiated $SAP^{-/-}$ recipients. Animals were challenged 8-10 weeks post- infusion with the T cell dependent antigen NP-CGG and analysis performed after 10 days. We demonstrated significant improvement in germinal centre formation

and NP-specific antibody responses with 20-40% engraftment of gene modified T cells. Using a SIN-lentiviral construct with codon optimised SAP transgene expression driven by the constitutive EFS promoter, we efficiently transduced XLP patient T cells resulting in improved cytotoxicity and T_{FH} cell function in vitro. In addition, using an LCL lymphoma model in NSG mice we demonstrated that adoptive transfer of gene corrected patient CTLs reduced tumour burden. Overall this data supports the further development of an autologous gene corrected T cell approach, which may offer an alternative therapeutic option for patients with XLP.

66. Expansion of Human T Regulatory Cells by Lentiviral Vector Mediated Expression of STAT5B or BACH2 Transcription Factors

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It has been recently shown that HIV-1 insertions targeting the transcription factors BACH2 and STAT5B are enriched and persist for decades in hematopoietic cells from patients under Anti-Retroviral Therapy (ART), indicating that insertional mutagenesis provided a selective advantage to these cell clones.

We specifically identified that chimeric mRNA transcripts containing viral HIV-1 sequences fused by splicing to the first protein-coding exon of STAT5B or BACH2 are present in the peripheral blood mononuclear cells (PBMC) of 30 out of 87 (34%) patients under ART. These chimeric mRNAs, putatively encoding for unaltered versions of BACH2 or STAT5B, were found to be specifically enriched (>10 fold, $p<0.001$) in T regulatory (Treg) cells in all patients tested (N=9) as the result of a selection mechanism triggered by promoter insertion, a well-known phenomenon induced also by genotoxic lentiviral vector (LV). Given that HIV-1/STAT5B and HIV-1/BACH2 transcripts were specifically found in Treg cells collected 6 years apart from the PBMC previously analyzed, these data suggest that HIV-mediated transcriptional activation of these transcription factors provide a long lasting selective advantage to Treg cells in HIV infected patients. LV-mediated expression of the wild-type form of STAT5B and BACH2 in Treg cells purified from healthy donors did not alter their phenotype and functions in vitro and significantly increased their proliferative capacity in competitive proliferation assays ($p<0.0001$). Moreover, co-injection in NSG mice of GFP-, BACH2- (N=7) and STAT5B (N=7) -transduced Treg cells with human allogenic PBMCs was able to prevent xenogeneic graft versus host disease in 75% of treated mice (N=4 for GFP, N=7 for BACH2 and N=7 for STAT5B Treg cells). Additionally, mice receiving STAT5B-over-expressing Treg cells showed a significantly reduced level of the overall human chimerisms ($p<0.001$) in the blood when compared to mice treated with GFP-overexpressing Treg cells, suggesting a superior activity of STAT5B-expressing cells in controlling the expansion of human PBMC.

Hence, beside its implication in HIV biology, the proliferative effect conferred by the STAT5B and/or BACH2 overexpression in Treg cells could represent a novel suitable approach for adoptive immunotherapy clinical application. Indeed, for such clinical purposes high number of cells are required, and the ex vivo transduction of purified Treg cells with LV expressing BACH2 or STAT5B should led to higher yield of Treg cells over other protocols of expansion and should promote the persistence of the transduced Treg cells in vivo.

67. Development of Gene Editing Strategies Aimed at Inducing Fetal Hemoglobin for the Treatment of Hemoglobinopathies Using the Nonhuman Primate Model

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Allogeneic hematopoietic stem cell (HSC) transplantation is currently the only existing cure for β-hemoglobinopathies but is not accessible to most patients and is associated with significant morbidity. Reactivation of fetal hemoglobin (HbF) production in adults constitutes an alternative therapeutic approach and the targeting of genes involved in HbF regulation has recently come under intense scrutiny. Here, we establish a nonhuman primate (NHP) transplantation model to evaluate gene editing strategies aimed at increasing HbF production for the treatment of hemoglobinopathies. We first characterized a transient HbF induction following myeloablative autologous HSC transplantation in NHP, which was comparable in duration in all treated animals but varied in amplitude, similarly to the response documented in human patients. As proof of concept, we targeted the repressor of HbF, BCL11A, in NHP HSCs by electroporation of TALE nuclease mRNA. Engraftment of Bcl11a edited CD34+ cells was low but we were able to track Bcl11a mutation signatures for over 200 days after transplantation confirming the persistence of Bcl11a-edited cells after transplantation. The initial transplant-associated HbF induction was comparable to controls but HbF eventually reached a set point that was significantly greater than control levels and persisted for over 2 years of follow up. Since the ubiquitous inactivation of Bcl11a may have adverse effects on non-erythroid cell lineages, we then turned to genetic alterations identified in individuals with hereditary persistence of fetal hemoglobin. In particular, recent evidence demonstrated that a 13-nucleotide deletion in the promoter of the *HbG1* gene increased HbF expression in human cells *in vitro*. We confirmed that the function of this DNA sequence is conserved in NHP by CRISPR/Cas9-induced mutagenesis. Up to 35% editing efficiency was achieved in NHP HSCs with minimal deleterious effect on multilineage colony-forming potential. In vitro erythroid differentiation of these cells demonstrated increased HbF expression that positively correlated with editing efficiency. To maximize the therapeutic potential of this approach, we successfully integrated a targeting cassette at the same site in NHP HSCs by co-delivery of CRISPR/Cas9 and an AAV homologous DNA template. Future experiments will investigate the long-term

engraftment of *HbG1*-edited HSCs and corresponding HbF induction in NHP transplantation experiments. In summary, the NHP model described here will prove valuable to evaluate the safety and efficacy of novel gene editing strategies aimed at treating hemoglobinopathies.

68. Cytoreductive Conditioning Intensity Predicts Clonal Diversity in ADA-SCID Retroviral Gene Therapy Patients

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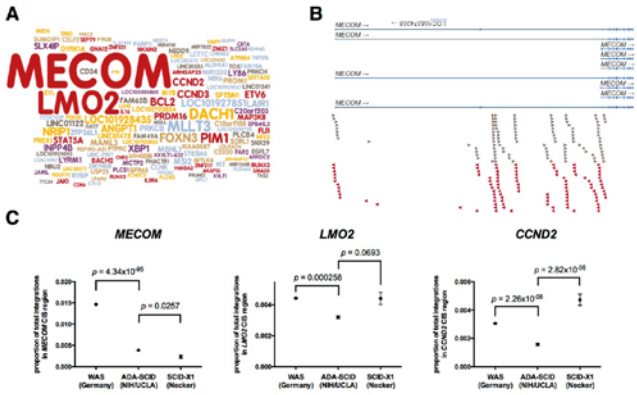
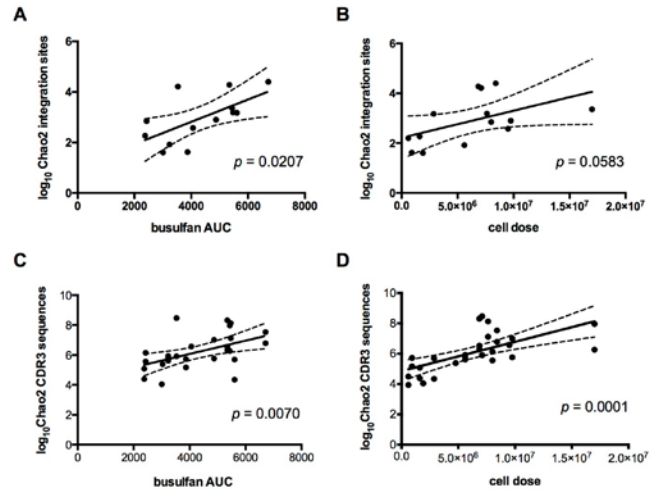
Retroviral gene therapy has proven efficacious for multiple genetic diseases of the hematopoietic system, but roughly half of clinical gene therapy trial protocols using gammaretroviral vectors have reported leukemias in some of the patients treated. In dramatic contrast, 39 ADA-SCID patients have been treated with four distinct gammaretroviral vectors without oncogenic consequence. We investigated clonal dynamics and diversity in a cohort of 15 ADA-SCID children treated with gammaretroviral vectors and found clear evidence of genotoxicity, indicated by numerous common integration sites near proto-oncogenes and by increased abundance of clones with integrations near MECOM and LMO2 . These clones showed stable behavior over multiple years and never expanded to the point of dominance or dysplasia. One patient developed a benign clonal dominance that could not be attributed to insertional mutagenesis, and instead likely resulted from expansion of a transduced NK clone in response to chronic EBV viremia. Clonal diversity and T-cell repertoire, measured by vector integration site sequencing and T-cell receptor beta chain rearrangement sequencing, both correlated significantly with the measured busulfan preconditioning dose, while T-cell repertoire correlated significantly with the CD34+ cell dose . These data, in combination with results of other ADA-SCID gene therapy trials, suggest that disease background may be a crucial factor in leukemogenic potential of retroviral gene therapy, and underscore the importance of cytoreductive conditioning in this type of gene therapy approach.





69. A Diversity of Human Hematopoietic Differentiation Programs Identified Through In Vivo Tracking of Hematopoiesis in Wiskott-Aldrich Syndrome Patients

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Several studies have highlighted murine hematopoietic stem cell (HSC) heterogeneity using single cell transplantation, clonal tracking barcoding analysis as well as RNAseq single cell analysis. Here we have used data from a gene therapy trial to treat Wiskott-Aldrich syndrome (WAS) to explore hematopoiesis in humans. In the trial, the therapeutic vector (lentivirus) integrates into the genome at unique positions in each hematopoietic stem and progenitor cell (HSPCs) and is consequently transmitted to all its progeny. Thus hematopoietic ontogeny in humans can be inferred by tracking the appearance of unique integration sites in fractionated blood cell populations. We concentrated on four WAS patients treated by gene therapy with two distinct sources of autologous HSPC : bone marrow (BM) or mobilized peripheral blood (MPB) (following administration of granulocyte

colony-stimulating factor (G-CSF)). In these patients, we have sorted peripheral blood samples for 5 cell types: myeloid (granulocytes and monocytes) and lymphoid subpopulations (T, B and NK cells), and analysed their IS profile (using our new optimized pipeline, INSPIIRED). Each IS corresponds to a particular stem/progenitor cell clone, for which we can quantify its contribution in each of the 5 lineages. Using this approach, we have characterized up to tens of thousands IS per patients, including two timepoints of follow up (1 y and 3 y) in order to study longitudinal dynamic. Statistical methods to account for sparse sampling and imperfect cell purifications comprise an important part of our approach and are under development. In initial analysis, using clustering algorithms, we identified different groups of IS clones corresponding to different human hematopoietic differentiation programs. We showed that a significant fraction of IS clones are detected in a single lineage, while other IS clones are characterized by different levels of contribution to the myeloid and lymphoid lineages, highlighting the heterogeneity of human HSC. Clones contributing to all 5 lineages are readily recovered but this study also unravels a diversity of inferred hematopoietic programs with various potentials contributing to human blood homeostasis. Longitudinal analysis of clonal dynamics is ongoing, with preliminary results showing the maintenance of this heterogeneity of HSPC over time. We will also present the differences of hematopoietic programs observed between the two sources of HSPCs (BM or MPB). These new findings and approaches suggest the existence of various types of human HSPC and provide unique data on human hematopoiesis.

Oligonucleotide Therapeutics

70. Mechanism and *In Vivo* Activity of a Small Activating RNA Targeting CEBPA, a Novel Therapeutic in Clinical Trials for Liver Disease

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Small activating RNAs (saRNAs) are short double-stranded oligonucleotides that are designed to selectively increase gene transcription. Previously we designed an saRNA that upregulates the transcription factor CCATT/enhancer binding protein alpha (CEBPA). We have now developed this saRNA into a clinical candidate, CEBPA-51, and here we investigate its mode of action and activity in an *in vivo* rat model of liver failure.

A nucleotide walk performed around bioinformatically-derived hotspots of saRNA activity in the CEBPA gene identified the sequence for CEBPA-51. This saRNA upregulates CEBPA mRNA 2.5-fold and C/EBP-α target gene albumin by 2.3-fold in human hepatocellular carcinoma HepG2 cells. A nuclear run-on assay confirmed that this is a transcriptionally-driven process. Mechanistic experiments demonstrate that Ago2 is required for saRNA activity, with the guide strand of the saRNA duplex shown to be associated with Ago2 and localized at the CEBPA genomic locus using RNA ChIP assays. Mutations in the seed sequence of CEBPA-51 caused a loss of activation, supporting a sequence-specific on-target saRNA activity of CEBPA-51. CEBPA-51 has been formulated in SMARTICLES® nanoparticles (MTL-CEBPA) for liver delivery and when administered at 4 mg/kg over 2 weeks leads to 90% inhibition of tumor growth in a diethylnitrosamine-induced cirrhotic liver cancer model and improvement in liver function. This novel drug is currently in a Phase I clinical trial for patients with liver cancer, and this represents the first human study of a saRNA therapeutic.

To investigate the activity of MTL-CEBPA in a model of fibrosis and acute liver failure, rats were exposed to CCl4 for up to 35 weeks. During this time animals were treated with either short term (2 weeks) or long term (14 weeks) MTL-CEBPA starting at week 8. We observed a dramatic improvement in liver function with restoration of AST, ALT, ammonia, and prothrombin time to near normal levels after just 2 weeks of MTL-CEBPA treatment. This was accompanied by highly significant reduction in hydroxyproline levels and other markers of liver fibrosis/inflammation. CEBPA mRNA expression in the liver as well as serum albumin were significantly elevated, demonstrating target engagement. At week 35, a significant increase in survival was observed where only 2 of the 9 rats deceased in the MTL-CEBPA group. No animals survived in the control group. These data strongly support exploring the additional clinical development of MTL-CEBPA for liver cirrhosis.

71. A 3' tRNA Derived Small RNA (tsRNA) Affects Translation in Rapidly Dividing Cells and a Target for Hepatocellular Carcinoma

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There are tens of thousands of different tRNA-derived small RNAs (tsRNAs) of 18-40 nucleotides in length in mammalian cells. In recent years there is accumulating evidence suggesting that these RNAs can play different yet important roles in gene regulations. Nevertheless, in most cases, the biological roles of these RNAs have yet to be defined. Using a variety of locked nucleic acid/antisense oligonucleotide (LNA/ASO)-mediated strategies, we found that inhibition of one specific 3'tsRNA, induces apoptosis in rapidly dividing cells. Inhibition of the tsRNA but not the mature tRNA reduced the translation of ribosomal protein S28 (RPS28) mRNA and led to a block in pre-18S ribosomal RNA processing, and ultimately a decrease in the number of 40S ribosomal subunits. Using a modified ChIRP method, we found that the tsRNA binds to ribosomal protein S28 mRNA and by genetic

complementation analyses established two binding sites contained within the mRNA. Furthermore, we established that the binding of the tsRNA to these sites were required for optimal translation. The systemic delivery of a specific anti-tsRNA oligonucleotides into mice (the tsRNA sequence is the same in mouse and humans) did not cause liver injury in normal mice but induced apoptosis and a significant growth retardation of patient-derived orthotopic hepatocellular carcinomas surgically implanted into mouse livers. Our result establishes a newly defined post-transcriptional mechanism of gene regulation and provides a novel target for cancer therapeutics.

72. TLR9-Targeted Systemic Delivery of CpG-STAT3 Antisense Oligonucleotides Induces Regression of Bone-Localized Prostate Tumors in Mice

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Signal Transducer and Activator of Transcription 3 (STAT3) is an oncogenic transcription factor, which plays important role in both prostate cancer progression as well as in sustaining immune-suppression in the tumor microenvironment. We previously demonstrated that Toll-like Receptor 9 (TLR9) ligands allow targeted delivery of oligonucleotides to $TLR9^+$ cells in prostate tumors, such as cancer stem-like cells and tumor-associated myeloid immune cells. Here, we describe new strategy to deliver nuclease-resistant STAT3 antisense oligonucleotides (ASO) to bone-localized prostate cancer. Tethering TLR9 agonist (CpG-ODN) to STAT3 ASO permits internalization of the CpG-STAT3ASO conjugate by $TLR9^+$ human and mouse cells without transfection reagents. We demonstrate that CpG-STAT3ASO is internalized by polymorphonuclear myeloid-derived suppressor cells (PMN-MDCSs) derived from blood of prostate cancer patients, as well as certain human (DU145, LN-TLR9) and mouse (Myc-CaP, Ras/Myc-driven RM1/9) prostate cancer cells. Target gene knock down by CpG-STAT3ASO had accelerated kinetics at mRNA and protein levels compared to the STAT3ASO alone. The biodistribution studies in mice showed that intravenous injections of $CpG\text{-}STAT3ASO^{Cy3}$ effectively targeted $TLR9^+$-myeloid cells in spleen and bone marrow. For efficacy studies, we used mouse syngeneic RM9 and $Pten^{pc-/-}Smad4^{pc-/-}Trp53^{c-/-}$(PST) models of castration-resistant prostate tumors implanted intratibially. Repeated *i.v.* injections of unformulated CpG-STAT3ASO (5 mg/kg) induced regression of bone-localized of tumors in the majority of treated mice. Antitumor effects of CpG-STAT3ASO depended on combination of direct and immune-mediated cancer cell killing as suggested by reduced efficacy in $Tlr9^{-/-}$ mice and in immunodeficient NSG mice. In immunocompetent mice, CpG-STAT3ASO treatment reduced STAT3 activity in both cancer cells and in tumor-associated immune cells, thereby reducing PD-L1 levels on $CD11b^+Gr1^+$ MDSCs together with the percentage of $CD4^+FoxP3^+$ regulatory T cells in tumor-draining lymph nodes. Our preliminary *in vitro* studies support translational potential of this strategy. Primary human PMN-MDSCs incubated with CpG-STAT3ASO showed reduced immunosuppressive potential, thereby restoring proliferation and activity of co-cultured T cell. We believe that our strategy can generate two-pronged targeting of metastatic, castration-resistant prostate cancers using safer and more efficient reagents based on TLR9-targeted oligonucleotide delivery.

73. Treatment of Sepsis by Neutralization of Extracellular Histones with Nucleic Acid Aptamers

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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, coagulation abnormalities, and increased endothelial permeability, leading to multiple organ dysfunction syndrome (MODS) and acute respiratory syndrome (ARDS). Recent evidence suggests that the molecular mechanism responsible for MODS/ARDS associated with sepsis involves extracellular histones. Histones are normally present in the nucleus of eukaryotic organisms. However, apoptotic and necrotic cells, and/or neutrophil extracellular traps (NETs), release histones into the extracellular space. Once in the extracellular fluid, histones activate toll-like-receptor (TLR) pathways and increase cellular Ca^{2+} influx, resulting in platelet aggregation, endothelial cell activation, and cytokine release. This self-propagating tissue injury is a significant contributor to development of MODS/ARDS, for which there is currently no treatment other than supportive care and a mortality rate approaching 40%. We hypothesized that neutralization of extracellular histones with nucleic acid aptamers (anionic molecules) can prevent the morbidity and mortality associated with sepsis. We have employed Systemic Evolution of Ligands by Exponential Enrichment (SELEX) technology to identify RNA aptamers that bind with high affinity (low nM-pM range) and specificity to those histones (H3 and H4) known to cause MODS/ARDS but not to other proteins present in blood or on cells. We confirmed that histones H3/H4 induce pronounced platelet aggregation, which can be inhibited by the addition of the selected RNA aptamers. Furthermore, we demonstrate that histone-induced cytotoxicity can be reversed by treatment with the RNA aptamers both *in vitro* (lung-derived endothelial and epithelial cells) and *in vivo* in a mouse model of MODS/ARDS. Current efforts are focused on evaluating and the efficacy and safety of these RNA bio-drugs in other established murine models of sepsis (e.g. cecal ligation and puncture). In conclusion, we present robust preclinical data on a novel class of therapeutics against circulating histones that may be potentially effective in a common clinical condition with high degree of morbidity, mortality and expense and for which, there is currently no effective treatment thus, establishing a paradigm change in the treatment of septic patients.

74. Modulation of Pro-Inflammatory IL-6 Trans Signalling Axis by Splice Switching Antisense Oligonucleotides as a Therapeutic Modality in Inflammation

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Interleukin-6 (IL-6) is a pleiotropic cytokine that plays a key role in maintaining homeostatic process to pathogenesis of various inflammatory and autoimmune diseases. This context dependent effect from the cytokine is due to two distinctive forms of signalling: *cis*- and *trans*-signalling. Various strategies have been employed in the past decade to target the pro-inflammatory effect of IL-6 in numerous inflammatory disorders, however their development have been hindered due to the fact that, these approaches target global IL-6 signalling, hence affecting the anti-inflammatory pathways of IL-6 signalling. Therefore, novel strategies that specifically targets the pro-inflammatory IL-6 *trans*-signalling without affecting IL-6 *cis*-signalling are needed and carry immense therapeutic potential. Following this path, we have developed a novel strategy to specifically decoy IL-6-mediated *trans*-signalling, by modulating alternative splicing of IL-6 signal transducer (Gp130) by employing splice switching oligonucleotides (SSO), to induce a truncated soluble isoform of Gp130. These soluble isoforms are devoid of signalling domains, but retains binding domains, which would allow to specifically sequester IL-6/sIL-6R receptor complex with high affinity in serum and thereby suppress inflammation.

We demonstrate that, Gp130 alternative splicing can be modulated to generate soluble antagonist isoforms, which can bind to IL-6/sIL-6R heterodimeric complexes and downregulate STAT3 activation. To further translate this approach to pre-clinical scenarios, we have utilised a neutral antisense oligonucleotide chemistry, phosporodiamidate morpholino oligomer (PMO), which was proven to be non-toxic compared to conventional charged ON analogues. For efficient delivery to cells the SSOs was conjugated with the Pip6a cell penetrating peptide (Pip6a-PMO). Furthermore, upon systemic administration in wild-type mice, a single dose of 15mg/kg Pip6a-PMO induced potent exon skipping in most of the tissues including liver, lungs, GI tract and prominently in the heart and muscles, without any apparent signs of toxicity. Of note this is the first study, where body-wide biodistribution of CPP-PMO conjugates has been addressed on functional level and highlights the global functional uptake of Pip6a-PMO conjugates. To determine the therapeutic potential of these novel SSO compounds in down regulating inflammation we have used the LPS induced acute inflammation model in mice, which mimics sepsis. Upon treatment of the animals with Pip6a-PMO targeting Gp130, we observed a 3 fold down regulation in IL-6 levels as compare to control group, in addition improved survival was also observed at 24 hours. These observations clearly show the therapeutic potential of this strategy to treat inflammatory disorders.

75. Artificial miRNAs Reduce Human Huntingtin Throughout the Striatum in a Transgenic Sheep Model of Huntington's Disease

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Huntington's disease (HD) is a devastating and fatal neurodegenerative disease caused by a genetic expansion of the CAG repeat region in the *huntingtin* (*HTT*) gene. RNA interference has emerged as a leading candidate disease modifying therapeutic for HD. Numerous studies have shown that artificial miRNAs can reduce mutant huntingtin in rodent models of the disease. The HD transgenic sheep are currently the only large animal model expressing the full-length human huntingtin. These sheep express a human huntingtin cDNA from the human HTT promoter as well as the normal endogenous sheep huntingtin. The human CAG repeat region comprises 73 glutamines encoded as 69 pure CAG repeats and the penultimate CAACAGCAACAG. We used an AAV9 vector to deliver an artificial miRNA targeting exon 48 of the human Htt mRNA unilaterally into the striatum of sheep. We examined the extent of silencing of human Htt using artificial miRNAs under the control of two alternative promoters. One month post-injection, AAV9-U6-anti-HTT reduced human huntingtin mRNA and protein in the striatum by between 70-80%. At six months, mRNA and protein were still reduced up to 50%. AAV9-CβA-anti-HTT reduced human huntingtin mRNA and protein up to 50% at one month and up to 60% at six months. Silencing was detectable throughout much of the caudate and putamen. We used immunohistochemistry and immunofluorescence to examine the effect of treatment on cells in the striatum. We observed an increase in activated IBA-1 positive microglia on the injected side one month after injection in both AAV-miRNA treated and control treated groups. At six months, there was no difference between injected and non-injected sides. This suggests that there is a transient increase in inflammation after surgery, which resolves over time. At six months post-injection, there was no significant loss of DARPP32 or NeuN labelled cells on the injected side compared to the non-injected side in any of the treatment groups. We conclude that safe and effective silencing of human HTT can be achieved in a large animal brain by direct delivery of an AAV carrying an artificial miRNA.

76. Small Hairpin RNAs Delivered in Human Cortical Spheroids Compete with Endogenous microRNAs

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The success of RNA interference (RNAi) therapeutics depends on sustained expression of delivered small hairpin RNAs (shRNAs) and sufficient gene knockdown without overloading the endogenous microRNA machinery and causing toxicity. This toxicity has been demonstrated in various tissues, most notably the liver and the brain. We have previously demonstrated using recombinant adeno-associated viral (rAAV) vectors expressing shRNAs that the toxicity results from shRNA competition with miR-122-5p microRNAs leading to de-repression of miR-122 target mRNAs in the liver. There is no effect on other microRNAs. Herein we extend our findings to identify the consequence of high levels of shRNA expression in a human 3D cellular model of the cerebral cortex derived from induced pluripotent stem cells - cortical spheroids. These floating 3D spheroids grow up to 5 mm in diameter and include synaptically connected deep- and superficial-layer pyramidal neurons and non-reactive astrocytes. Transcriptionally, the spheroids resemble the late mid-fetal human cortex. Through the delivery of rAAV vectors expressing shRNAs, we determine that high shRNA levels exceeding 12% of shRNA plus microRNA reads lead to decreased viability of plated neurons. The reads specifically compete with miR-9, one of the most abundant microRNAs in this model, though not with other abundant brain microRNAs such as miR-124 and miR-125. The consequences on miR-9 expression recapitulate several of the cardinal features we observe for miR-122 in the liver including competition with the first synthesized isoform of each microRNA. This data builds towards a unifying mechanism behind the consequence of exogenous shRNA expression on endogenous microRNAs in specific tissues with adverse responses to excessive shRNA levels, and has potential implications for gene therapy for disorders of the nervous system.

RNA Virus Vectors

77. Lentiviral Vectors Escape Innate Sensing but Trigger p53 in Human Hematopoietic Stem and Progenitor Cells

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Encouraging clinical results of recent hematopoietic stem and progenitor cell (HSPC) gene therapy trials are opening novel perspectives for the treatment of monogenic diseases affecting the hematopoietic system. In this setting, self-inactivating (SIN) lentiviral vectors (LV) have been successfully used in clinical trials for the treatment of several diseases. Nevertheless, LV induced signaling and its potential functional consequences on HSPC biology remain poorly understood. To address this, we have performed a transcriptome analysis on human HSPC exposed to VSV-g pseudotyped LV at a high multiplicity of infection, matching current clinical vector dose requirements. We unravel a remarkably limited impact of LV on the HSPC transcriptome. LV efficiently escaped innate immune sensing that instead led to robust type I IFN responses upon transduction with a gamma-retroviral vector. However, LV transduction did trigger DNA damage responses in human HSPC of different sources, cord blood as well as the more clinically relevant bone marrow, and mobilized peripheral blood (mPB). In particular, p53 signaling was among the most significantly altered pathways ($p<1.03x10^{-12}$) and induction of several key players, including an 8-fold increase in p21 mRNA, was further confirmed by Taqman. LV-mediated triggering of p53 depended on efficient nuclear import of reverse-transcribed viral DNA but did not require integration as it occurred also using an integrase-defective LV and with a non-integrating Adeno-associated vector (AAV6). Direct p53 inhibition through the overexpression of a dominant negative peptide of p53 completely blocked p21 mRNA induction upon LV and AAV6 transduction, further indicating that p53 is a key modulator of these responses in HSPC. Functionally speaking, LV-mediated signaling led to a slight delay in HSPC proliferation, increased apoptosis in culture and reduced engraftment capacity *in vivo*. Induction of p53 signaling and subsequent decrease in engraftment was also confirmed in mPB-$CD34^+$ cells transduced with a clinical-grade LV according to the current gold standard transduction protocol, corroborating the clinical relevance of our findings. These effects were more pronounced in the short-term repopulating cells while long-term HSC frequencies remained unaffected. Blocking LV and AAV6-induced signaling through pharmacological inhibition of the p53 upstream activator ATM partially rescued both apoptosis and *in vivo* engraftment, with minimal impact on the cell proliferation delay. These results suggest that a window of non-apoptotic quiescence that favors engraftment can be reached in these conditions and highlight a novel strategy to further dampen the impact of *ex vivo* gene transfer on HSPC. Overall, our results shed light on viral vector sensing in HSPC and provide critical insight for the development of more stealth gene therapy and gene correction strategies.

78. LTR1 Vectors Enhance Safety in Gene Therapy and Can Be Exploited for Rapid, Transient Gene Delivery

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Lentiviral vectors require more than 19% of the wild-type HIV-1 genome to be present in their genomic RNA for efficient packaging into viral particles. As a consequence, lentiviral proviruses normally contain the HIV-1 packaging signal, the major HIV-1 splice sites, the REV-response element (RRE) and clusters of CpG islands. These features carry a risk of adverse effects in transduced cells, such as generation of patient-vector fusion transcripts, transgene silencing due to methylation of viral CpG islands and mobilization of vector genomes in HIV-1 particles.

These issues can potentially be avoided with our novel lentiviral vector, LTR1, in which the unwanted HIV-1 packaging sequences are repositioned to exclude them from reverse-transcription and prevent their incorporation into the DNA provirus. We report that this property renders LTR1 resistant to remobilization in mock HIV-1-infected cells.

The presence of HIV-1 splice donor and splice acceptor sites in 3rd generation lentiviral proviruses has been linked to the generation of aberrant vector-patient fusion transcripts, but LTR1 proviruses are devoid of the major HIV-1 splice sites. To examine the effect of this, we generated RNAseq libraries from cells transduced with either LTR1 or 3rd generation vectors and quantified vector-cell genome fusion transcripts using standard bioinformatic tools. Importantly, LTR1 proviruses reduced fusion transcripts to 13% of the level produced by 3rd generation proviruses.

We show that LTR1 technology can be used for preclinical gene therapy. LTR1 or a 3rd generation vector was used to deliver factor IX cDNA to Factor IX deficient mice by intravenous administration. At 67 days post-injection, mouse plasma Factor IX activity was raised to 14.8% by LTR1, matching the 3rd generation vector level of 12.6%, showing that LTR1 technology is competitive in a gene therapy setting.

We also report a functional advantage of LTR1 in its rapid onset of transgene expression. The LTR1 structure lends itself to this application, given the absence of a 5' leader sequence. We show that this property can be harnessed, in combination with a reverse-transcriptase-deficient configuration, to promote delivery of LTR1 genomic RNA as mRNA for efficient, transient gene delivery to HEK 293T cells. In our investigation, vector expression was strong by 4 hours post-transduction and peaked at 24 hours. At day 13, harvested genomic DNA was completely devoid of vector proviruses.

In all, our recent data have highlighted potential safety advantages of LTR1 in its resistance to remobilization and reduced splicing into target cell genes, whilst our *in vivo* data show that these safety advantages would not come at the expense of therapeutic efficacy. Finally, we have shown that the structure of LTR1 can be exploited for rapid, transient delivery of transgenes as mRNA, which could provide an important tool in gene editing and stem cell manipulation.

79. Generation of Immune Stealth Lentiviral Vectors by Producer Cell Genome Editing

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Lentiviral vectors (LV) are powerful and versatile vehicles for *ex vivo* and *in vivo* gene therapy. However, their complex biological composition challenges large-scale manufacturing and raises concerns for in vivo applications, because particle components and contaminants may trigger innate and adaptive immune responses that may cause toxicity and jeopardize gene transfer in the recipient. Here, by editing the genome of producer cells, we modified the protein composition of the vector envelope and obtained novel LV with preserved infectivity but substantially reduced immunogenicity and increased resistance to phagocytosis by human macrophages *ex vivo* and *in vivo* in mice. We performed genetic disruption of the β-2 microglobulin (B2M) gene, a required component for the assembly and trafficking of all class-I major histocompatibility complexes (MHC-I) to the plasma membrane in LV producer cells, exploiting the RNA-guided Cas9 nuclease. The resulting B2M-negative cells were devoid of surface-exposed MHC-I and produced MHC-free LVs. These LVs retain their infectivity on all tested cells *in vitro* and efficiently transduced the mouse liver upon intravenous administration. These MHC-free LVs showed significantly reduced immunogenicity in a T-cell activation assay performed on human primary T cells co-cultured with autologous monocytes exposed to LV, from several healthy donors, suggesting that conventional MHC-bearing LV may trigger allogenic immune responses. Moreover, we have generated LV with increased levels of CD47, a phagocytosis inhibitor, on the vector surface, which show decreased uptake by human macrophages *in vitro*. In order to evaluate the role of CD47 in LV biodistribution upon *in vivo* administration we took advantage of the non-obese diabetic (NOD) mouse model whose SIRPα (the CD47 receptor) is known to have high affinity for the human CD47. In this setting, CD47 proved to be a key player in extending the circulating half-life and reducing macrophage uptake of LV and decreasing the inflammatory cytokine response following their administration. Altogether these improved LV may be advantageous for *in vivo* delivery in reducing innate and adaptive immune response to the gene transfer. We also applied these improvements into a novel inducible packaging cell line, which allows consistent generation of LV producers upon site-specific integration of the vector genome of interest. Overall, these advances support scalable manufacturing of LV with higher purity and immune stealth features that should improve the safety and efficacy of LV in human gene therapy.

80. BaEV-LVs Efficiently Transduce HSCs-Derived Human Progenitor T Cells, Acceleratingt Cell Reconstitution In Vivo and Allowed Correction of X-SCID Progenitor T Cells

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T cells represent valuable tools for treatment of cancers, infectious and inherited diseases but their use in innovative therapies is currently limited because they are mainly short-lived in vivo. T-cell based therapies would strongly benefit from gene transfer into live-long persisting T cells in vivo. Therefore, we initially compared the gene transfer efficiency into early progenitor T cells isolated from human thymus with a new lentiviral vector (LV) pseudotyped with the baboon retrovirus envelope (BaEV) to vesicular stomatitis Virus G enveloped LVs (VSVG-LVs). Equivalent transduction levels were revealed for BaEV-LVs in freshly isolated early thymic progenitors (ETP), progenitor and pre T cells from human thymus reaching 80% transduction, outperforming conventional VSVG-LVs (10%). A promising therapeutically feasible alternative, is the use of T cell progenitors, which can be generated in large amounts upon exposure of human CD34+ cells to the Notch ligand DL-4. We showed here that these in vitro generated ETPs and T progenitors were transduced with BAEV-LVs up to 80-90% and allowed efficient T cell reconstitution of NOD/SCID/gammaC$^{-/-}$(NSG) mice in vivo in all the hematopoietic tissues. They maintained these high transduction levels in all derived T cell subpopulations and they permitted an accelerated T-cell lineage reconstitution and maturation as compared to CD34+ HSCs in the NSG mouse model. In X-linked severe combined immunodeficiency (X-SCID) the patients suffer upon transplantation of gammaC corrected CD34+ cells from a delayed T cell reconstitution during several months. To close this gap in T cell development, progenitor T cell therapy could be a valid option. Importantly, the BaEV-LVs encoding for gammaC, corrected X-SCID CD34+ derived T cell progenitors efficiently, as shown by efficient restoration of T cell development in vitro. These results indicate that BaEV-LVs are valuable tools for genetic modification of early T cell lineages, essential targets for gene therapy application where long-lived T cell persistence is important for durable treatment/correction of patients. Additionally, coinjection of LV-corrected autologous T cell progenitors and HSCs might accelerate T cell reconstitution in patients as compared to solely injecting HSCs avoiding a gap in immune reconstitution.

81. Multidimensional Vector Interaction Capture to Study the Effects of SIN.LVs and Chromatin Insulators on the Cellular Genome

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To date, the impact of Lentiviral Vector (LV) genomic integrations on the chromatin architecture of the host cell, especially when carrying strong enhancer-promoters or chromatin insulator (CI) that might be able to interact with distant genomic sequences, has not been extensively addressed. To study the interactions of LV integrations with the cellular genome, we set-up an *ad hoc* LV-specific Circular Chromosome Conformation Capture (LV4C) protocol able to retrieve the genomic portion flanking the LV integration site (IS) attached by proximity ligation to the corresponding host-genomic interaction site (ITS).

The LV4C protocol was applied to study the LV/genome interactions in 3 K562 cell clones harboring >60 IS (identified by Linear Amplification Mediated -LAM- PCR) of either a SIN.LV with the strong Spleen Focus Forming Virus enhancer-promoter (SIN.LV) or its insulated-version (CI.SIN.LV, two clones) containing a human CTCF-based CI sequence within the LTRs and untransduced K562 cells as control. As internal 4C control, we assayed for all samples the interactions on a validated gene (*MYC*) and found a high correlation (r=0.78) with published interactions obtained by HiC, validating the technical strength of our protocol.

LV/genome ITS found from all different clones were >1000. The correlation between IS/ITS and HiC data was high (r=0.61) indicating that LV/genome ITS occurred predominantly in existing host chromatin loops. IS as well as ITS distribution followed the conventional LV integration pattern, being mostly present inside gene rich regions with no skewing for the transcription start site. We classified different groups of ITS dependently on their distance from the IS and identified: self-ligations, capturing the vector IS (mostly matching those previously identified by LAM PCR); close ITS at an average distance of 5-10 kb; distal ITS, with an average distance of 100kb, and ITS far apart from the integration site, >100kb to 1Mb. Interestingly, we observed that 10% of the IS of the SIN.LV displayed long-range ITS, while such interactions for CI.SIN.LV raised to 20%. Of the interacting integration sites, approximately 85% had one ITS targeting a close or distal interaction cluster, while 15% had multiple interactions to these clusters. Interestingly, in an exploratory analysis we have identified genomic CTCF sites close to CI.SIN.LV interaction targets in 20% of the cases present in the correct orientation which could potentially generate CTCF-mediated chromatin loops between host genome and vector-contained insulators. Further validation of these interactions coupled to gene expression analysis of the genes inside vs. outside the chromatin loops will be instrumental to address the molecular mechanisms guiding insulation and the functionality of chromatin insulators in CI.SIN.LV constructs as well as the impact of lentiviral integration on host chromatin architecture and safety.

82. Library Screening for Novel Chromatin Insulator Elements for Use in Gene Therapy

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Insertional activation of proto-oncogenes represents a major safety concern in gene therapy with integrative vectors. One approach to reduce or eliminate inadvertent transcriptional activation of oncogenes is to include chromatin insulators in the vectors. Many known insulators require binding of CTCF to function and more than 30,000 CTCF binding elements have been identified in human genome, but very few have been tested for use in gene therapy vectors. In this regard, a key feature of insulators is the ability to block enhancer-mediated activation of cellular promoters, and most chromatin insulators only have partial enhancer blocking activity in heterologous cells. We have developed a genome-wide screen for enhancer blocking activity at a relevant proto-oncogene locus involved in prior vector-induced leukemia in immunodeficiency trials. We first recreated an oncogenic insertion in the LMO2 locus of a human Jurkat T cell line by inserting gamma-retroviral LTR-GFP cassette flanked by two loxp sites 3kb upstream of the transcription start site. A second targeting event introduced an Ires-GFP reporter cassette into the 3' UTR of LMO2 gene so that activation of LMO2 transcription can be detected by GFP expression. The Cre-lox recombination was then used to insert potential insulating DNA fragments between the LTR enhancer and the LMO2 promoter and insulator function was defined by levels of GFP expression in clones. Targeted insertion of a known cHS400 chicken beta-globin insulator reduced the GFP expression by 60%, while insertion of a control DNA element did not have effect on GFP expression, confirming that this cell line can be used to test and screen for insulator elements. We next constructed a CTCF-binding element plasmid library using SureSelect target enrichment method, which involved synthesizing RNA hybridization probes for 28,512 CTCF binding elements, sonication of Jurkat cell genomic DNA into 300-600bp fragments, hybridization and biotin-streptavidin pulldown of CTCF-binding elements and ligation of those elements into a plasmid. Deep sequencing of the plasmid library showed that it contains 13,597 unique human CTCF-binding elements. We then transfected the library into the Jurkat reporter cell line, along with a plasmid expressing Cre recombinase, to insert the CTCF-binding elements between the LTR and the LMO2 promoter, through Cre-mediated cassette exchange. The cells were cultured for two weeks and then sorted based on GFP expression into GFP-low and GFP-high subpopulations. Genomic DNA was extracted from GFP-low subpopulations and the inserted CTCF-elements were identified by deep sequencing. This screen identified 180 novel CTCF-binding elements that exhibited strong enhancer blocking activity, with several showing stronger enhancer blocking activity when compared to elements from the chicken B-globin cHS4 element. Three of these insulators have been subcloned into the U3 of the lentiviral vector CL20-MND-GFP and all three vectors showed titer of >$7x10^6$ tu/ml. In summary, we have identified a number of CTCF-binding elements from human genome that exhibits strong enhancer blocking activity and these elements may offer a safety advantage in human gene therapy, particularly in high risk immunodeficiency disorders prone to T-cell leukemia.

83. Gammaretroviral and Lentiviral Vector Based Insertional Mutagenesis Screens to Identify Prostate Cancer Therapeutic Targets

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Replication-incompetent gammaretroviral (γRV) and lentiviral (LV) vectors have both been used in insertional mutagenesis screens to identify cancer driver genes. In this approach the vectors stably integrate in the host cell genome and induce cancers by dysregulating nearby genes. The cells that contain a retroviral vector provirus in or near a proto-oncogene or tumor suppressor gene are preferentially enriched in a tumor. γRV and LV vectors have different integration profiles and genotoxic potential, making them potentially complementary tools for insertional mutagenesis screens. We performed insertional mutagenesis screens using both γRV and LV vectors to identify driver genes that mediate progression of androgen-independent prostate cancer (AIPC) using a xenotransplant mouse model. Vector transduced LNCaP cells were injected orthotopically into the prostate gland of immunodeficient mice. Mice that developed tumors were castrated to create an androgen-deficient environment and metastatic tumors that developed were analyzed. A high-throughput modified genomic sequencing PCR (MGS-PCR) approach identified the positions of vector integrations in these metastatic tumors. *TAOK3, MBNL2, SERBP1, SLC7A1, SLC25A24, MAN1A2, PLEKHA2, SPTAN1* and *ABCC1* were identified as candidate prostate cancer (PC) progression genes. Both *TAOK3* and *ABCC1*, identified by γRV and LV vectors respectively, increased clonogenicity *in vitro. TAOK3* and *ABCC1* expression in PC patients predicted the risk of recurrence after androgen deprivation therapy. Our data shows that γRV and LV vectors are complementary approaches to identify cancer driver genes which may be potential biomarkers and therapeutic targets.

Engineering and Manufacturing Vectors and Cells I

84. Novel Manufacturing of Gene Corrected Autologous Blood Stem Cells for Gene Therapy of Fanconi Anemia Complementation Group A

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A hallmark of the rare, monogenic disorder called Fanconi anemia (FA) is an accelerated decline in hematopoietic stem cells (HSC) leading to bone marrow (BM) failure. Long-term treatment requires bone marrow transplant from an unaffected donor, but success of this approach is limited for the ~70% of FA patients lacking matched sibling donors. Gene therapy could be an alternative, correcting the genetic defect in

the patient's own HSC and negating the need for a BM donor. Based on lessons learned in previous FA gene therapy studies, we developed an optimized protocol for lentivirus (LV)-mediated *FANCA* gene transfer into HSCs from FA-A patients [Becker, P. *Human Gene Therapy*, 2010]. This phase I clinical trial incorporates vector recommendations from the International FA Gene Therapy Working Group. Two patients have been treated on this trial to date (National Clinical Trials Registry ID: NCT01331018). In the first patient treated, a total of 3.2 x 10^7 CD34+ cells were present in 1.1L of BM, but only 9.4 x 10^6 total CD34+ cells were successfully isolated owing to dim CD34 expression. LV transduction at 10 infectious units (IU)/cell resulted in a vector copy number (VCN) of 0.33 per cell and 18.4% of colony-forming cells transduced. In the second patient, a total of 400mL of BM was collected, containing a total of 30.6 x 10^6 CD34+ cells. To avoid excessive CD34+ cell loss, the purification step was omitted and the entire red blood cell depleted BM product was subjected to LV transduction at 10 IU/cell. We observed a VCN of 1.83 per cell and 43% of colony-forming cells transduced, suggesting more efficient transduction of the mixed cell population. In addition, we observed greater viability of these cells during manufacture. These data suggest that avoidance of direct CD34 selection is advantageous for transduction and gene transfer in FA. To reduce vector needs and preserve available CD34+ cells for future FA patients, we developed a novel strategy to deplete lineage+ cells from both mobilized leukapheresis and bone marrow products. This process efficiently depletes >90% of CD3+, CD14+, CD16+ and CD19+ cells and retains a mean 94% of the initial CD34+ cell fraction in healthy donor BM (n = 8) and a mean 65% of the initial CD34+ cell fraction in mobilized leukapheresis products (n = 10). We demonstrate a mean 73% and 94% reduction in total nucleated cells in BM and leukapheresis products, respectively. Following transduction at 10 IU/cell, we observed mean vector copy numbers of 2.6 per cell in BM products and 5.4 per cell in apheresis products. Most importantly, we observed engraftment of lineage depleted cell products in an immune deficient mouse model equivalent to that of purified CD34+ cells from the same donor when transplanted at matched CD34+ cell doses. This novel selection strategy has been approved by the regulatory agencies with initial FA patient treatment planned for the first quarter of 2017.

85. Building a Better T-Cell: T Cell Process Development by Multifactorial Design of Experiments Approach and High Throughput Automated Functional Assays

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Adoptive T cell therapies, such as chimeric antigen receptor (CAR) modified T cells, have demonstrated promising clinical results in several indications. Antibody-Coupled T Cell Receptor (ACTR) represents a novel approach which enables a universal T cell product to be coupled with multiple types of antibodies targeting different tumor antigens. The potency of ACTR T cell products can be modulated by either varying the ACTR construct, or by controlling the T cell production process. Multiple reports have suggested the impact of changing the T cell manufacturing process on the potency of final T cell products. However, most of this process development work has been focused on a limited number of process parameters using a "one-factor-at-a-time" approach which neglects the interactions among multiple process parameters. In addition, the complexity of the functional assays used to evaluate different iterations of processes also limits the screening and optimization efficiency of a large number of process parameters and settings. Furthermore, it is still unclear which in vitro assays faithfully predict in vivo efficacy in preclinical animal models and ultimately efficacy in patients.

Here we present a systematic process development approach which incorporates a multifactorial Design of Experiments (DoE) method, combined with an automated high-throughput functional assay, mimicking chronic T cell stimulation within a tumor microenvironment (stress test). This approach was used to screen multiple parameters for critical T cell process elements, which in combination resulted in 70+ different processes. We were able to evaluate the impact of the changes to each process element individually as well as their synergistic effects. More importantly, based on this high-throughput process development method, we have developed unique combinations of several process parameters which have led to significant improvements of ACTR T cell function, as demonstrated by both in vitro and in vivo assays (preclinical human xenograft models in NSG mice). These unique process combinations are novel, and may not have been discovered using the traditional "one-factor-at-a-time" approach. The in vitro stress test offers a high throughput tool to efficiently screen a large set of samples.

Overall, we believe the multifactorial Design of Experiments approach, coupled with high throughput automated functional assays present a unique tool set for the process development and improvement of T cell therapy products.

86. *Sleeping Beauty* (SB) Transposition Integration Events Mediated by SB Transposase from mRNA and CAR from SB DNA for T-Cell Therapy

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The development of cost-effective and effective gene therapy requires (i) integration of desired transgene in the genome, (ii) long-term transgene expression without any unwanted immune response, and (iii) minimal risk of insertional mutagenesis. The non-viral *Sleeping Beauty* (SB) transposon system mediates chromosomal integration and stable gene expression when an engineered SB transposon is co-delivered with SB transposase. We conducted a phase I clinical trial of twenty six patients with advanced non-Hodgkin's lymphoma and B-cell acute lymphoblastic leukemia (B-ALL) infusing autologous and

allogeneic T cells genetically modified with SB DNA plasmids coding for transposon and transposase (SB11). The administration of CD19-specific T cells demonstrated the safety of the SB-modified CAR T cells, feasibility of this approach, and anti-tumor effect of chimeric antigen receptor (CAR). The infused SB-modified CAR T cells did not show any integration hot spots, persisted in both autologous and allogeneic recipients, and were not associated with any toxicity (Kebriaei et al, J Clin Invest. 2016;126(9):3363-3376). We have advanced the production process to generate CD19-specific CAR+ T cells with SB transposase introduced from *in vitro*-transcribed mRNA. As with our clinical trial, the SB-modified T cells were selectively propagated on activating and propagating cells derived from engineered K-562 cells. We characterized the resultant T cells for expression of CAR, integration events, redirected specificity, and insertion sites of the SB transposon. CAR integration site analysis by next generation sequencing was compared with the integration sites achieved when SB transposase was derived from DNA plasmid. As mostly observed with SB DNA transposase, unique insertion sequence data obtained with SB transposase mRNA showed (i) random insertion and 99.999% of the time at expected TA dinucleotide sites, with 33% of the insertions localizing to intragenic loci and the remainder 67% intergenic, (ii) the majority (97%) of intragenic insertions were intronic and the majority of intergenic transpositions fell within non-coding repeat regions, and (iii) no insertion within 1 Kb of the transcription start sites. Our data suggest that SB transposase derived from mRNA can be efficiently used to mediate stable non-viral gene therapy using T cells.

87. Destabilizing Domains Enable Inert and Consistent Regulation of GDNF Expression In Vivo

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Current regulatable expression systems have several shortcomings that prevent their use in a clinical setting. Our group has been working with a novel regulatable expression system based on destabilizing domains (DD). This version of DD uses the oral drug trimethoprim (TMP) to activate gene expression. We have used DD to regulate the expression of glial cell-line derived neurotrophic factor (GDNF) and shown that DD-regulated GDNF (GDNF-F-DD) was neuroprotective in an animal model of Parkinson's Disease and did not show any leakiness in the off state. Based on these promising results, we set out to fully characterise the DD system in animals. The first aim was to determine if GDNF-F-DD expression could increase in response to increasing concentrations of TMP. Lentiviral vectors (LV) expressing GDNF-F-DD were delivered to the striatum of Sprague -Dawley female rats and given increasing dosages of TMP in the drinking water. Three weeks after LV delivery the animals were euthanised, the striata dissected and processed for GDNF ELISA. Maximum expression of 66 pg/mg tissue of GDNF-F-DD was achieved when 0.2 mg/ml of TMP was given in the drinking water. The next aim was to ascertain how long was needed for GDNF-F-DD to be fully active in vivo and once fully activated, how long was needed for GDNF-F-DD to revert to uninduced levels. LV expressing GDNF-F-DD were delivered to the striatum of Sprague -Dawley female rats. The animals were given 0.2 mg/ml TMP in the drinking water for a period of 5 weeks after which they were switched back to regular drinking water. To ascertain GDNF-F-DD activity in the intended target cell population, immunohistochemistry for phosphorylated ribosomal protein S6 (pRPS6) was used. Quantification of pRPS6 positive cells indicated that GDNF-F-DD reached maximum activity 3 weeks after induction and GDNF-F-DD activity was lost 1 week after TMP withdrawal. We then set out to verify if GDNF-F-DD activity and expression could be maintained throughout multiple induction cycles. Again, LV expressing GDNF-F-DD were delivered to the striatum of Sprague -Dawley female rats. The animals were subjected to three cycles of TMP induction, where 0.2 mg/ml TMP was given three weeks for induction and withdrawn for 4 weeks. Quantification of pRPS6 positive cells indicated that GDNF-F-DD reached maximum activity in all three induction cycles with no apparent loss of activity. Furthermore, densitometric analysis for GDNF expression also indicated that similar levels of GDNF-F-DD were achieved in all three induction cycles. Lastly, we wanted to determine if prolonged expression of GDNF-F-DD would be toxic and lead to an inflammatory and immune response. LV expressing GDNF-F-DD, and control LV were delivered to the striatum of Sprague -Dawley female rats. One group of GDNF-F-DD animals were given 0.2 mg/ml TMP continuously. The animals were analysed 12 and 24 weeks after LV delivery. Quantitative analysis using NeuN and CD11B did not reveal any neurotoxicity or inflammation. Furthermore, there was no evidence of sustained immune response. Taken together, these results indicate that the DD system can robustly regulate gene expression over multiple cycles, has no off activity and is inert in vivo, making it a very promising system to regulate gene expression in a clinical setting.

88. S/MAR Based Nano-Vectors: Novel Tools for the Genetic Modification of Patient-Derived Cancer 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 cells such as primary human tumour cells are typically refractory to transfection with traditional bacterial plasmids that contain a large bacterial backbone comprising 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 genetically modifying patient derived (PDX) cells. This vector system provides autosomal replication and persistent and robust transgene expression in cells *in vitro* and *ex vivo* when xenografted into 'immuno-deficient' mice. This novel DNA nano-vector system is characterized by a minimally size bacterial backbone and an antibiotic free RNA-Out selection system. The presence of the S/MAR region prevents epigenetic silencing and provides persistent mitotic stability. We have

utilised this Nano-SMARt vector to genetically modify Patient-Derived Pancreatic Cancer cells using nano-vectors expressing the reporter gene GFP as well as SMAD4 - a crucial tumor suppressor gene that was lost during the tumourigenic development of these cancer cells. We show that the S/MAR nano-vectors remain as episomal replicons and provide robust transgene expression in these novel cell lines. We have xenografted the modified and unmodified cells into mice and evaluated the growth characteristics of the developing tumours. The novel cell lines we generated are essentially isogenic; the only difference between the genetically modified cell lines and the parental controls is the expression of the transgene. To demonstrate that the introduction of a Nano-SMARt vector does not affect the molecular and biochemical behavior of the cells we compared the growth characteristics and histological sections of the eGFP tumours compared to the parental cell lines and confirmed they were identical. Additionally, tyhe efficiency of this novel vector system was evaluated in subsequent experiments where we restored SMAD4 a key tumor-suppressor gene which had been lost during the malignant transformation of the originating pancreatic cancer cells. In comparative experiments we demonstrated that this process was not possible using our previous generation of plasmid-based DNA vectors. The success of the re-introduction of the gene was first tested by Western blot and its impact on the tumor development was determined in *in vivo* experiments. The cells were injected orthotopically into mice and the tumor growth was monitored over time. The modified cells generated a primary tumor which present clear differences in size and morphology compared to the parental control one. The effect of the tumor-suppressor gene in the biology of the pancreatic cancer is under investigation. Due to their robust and persistent expression and the non-integrative nature of this system these novel Nano-SMARt DNA vectors offer a powerful tool for the generation of isogenic cell lines which conserve the molecular behavior of the originating cells as well as providing a tool for introducing novel genetic modifications. These novel cells can be used for testing new therapeutic treatments and to better understand the cellular processes involved in tumor development.

89. RNA Aptamers Selected Through Blind-SELEX Inhibit Pancreatic Cancer Cell Metastasis and Invasion by Regulating Epithelial Mesenchymal Transition(EMT)

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Pancreatic cancer is known to be one of the most deadly cancers and ranked as the fourth cause of cancer related death in both Europe and United States. Mortality rate remains high because most patients are diagnosed with metastatic stage of disease at first diagnosed. Metastasis is the major cause of mortality in pancreatic cancer patients. There has been limited progress in therapeutic options for metastatic pancreatic cancer, and traditional chemotherapy outcomes, even though improved, are still disappointing. To improve the specificity and efficacy of therapeutic interventions, identifying and targeting proteins that are selectively exposed on the plasma membrane of tumor cells are essential. To this end, untargeted SELEX, also called "blind SELEX", was employed to generate highly enriched RNA aptamers against pancreatic cancer cells. This strategy allows us to identify aptamers that bind specifically to PANC-1 cells. To identify aptamer binding ligands which might be used as potential biomarkers, the cell membrane was retrieved using affinity purification through the RNA aptamer. The aptamer-bound proteins were then identified by tandem MS/MS (MASS-SPEC). The results of MASS-SPEC identified the target of P15 as vimentin, biomarker of epithelial mesenchymal transition (EMT), which is an intracellular protein but is specifically translocated on the plasma membrane of cancer cells. As EMT plays a pivotal role to transit cancer cells to invasive cells, tumor cell metastasis and invasion assays were performed *in vitro*. P15 treated pancreatic cancer cells showed the significant inhibition of tumor cell migration in wound healing assays and invasion in chemotaxis assay. To investigate the downstream effects of P15, 84 EMT related gene expression analysis was performed to identify differently expressed genes (DEGs). Among DEGs, P15 treated cells showed the down-regulated expression of matrix metallopeptidase 3 (MMP3), which is upregulated in EMT and involved in cancer invasion. In this study for the first time, we showed that P15 binding to tumor-associated vimentin inhibit the tumor cell metastasis and invasion throughout the down-regulation of MMP3. Our results suggest that P15 might be used as anti-metastatic therapeutics in pancreatic cancer.

90. Bioengineering Gene Therapy Vectors for Hemophilia A Through Ancestral Sequence Reconstruction

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Throughout evolution, functional proteins have emerged through diverse demands from physiological and environmental pressures. Within humans, the risk of thrombosis may have pressured coagulation factor VIII (FVIII) to be expressed at lower levels, have lower specific activity and/or be subjected to more rapid inactivation/clearance mechanisms. As human FVIII represents a transgene product incorporated into several clinical gene therapy candidates for hemophilia A, it may be possible to improve its pharmacological properties using information gained through the study of ancestral versions of the protein. Previously, we described ancestral sequence reconstruction (ASR) and characterization of ancient mammalian FVIII variants (Zakas *et al. Nature Biotechnology* 2017. 35, 35-37). One variant, designated An53, was shown to possesses several pharmaceutically desirable properties including improved specific activity, stability, biosynthetic efficiency, and reduced reactivity with anti-human FVIII monoclonal antibodies as well as inhibitor patient plasmas, despite having only 5% non-human FVIII sequence. In the current study, we further characterized pharmacological properties of An53 and investigated its potential in preclinical gene therapy studies. An important property of FVIII, which governs its circulating

half-life, is binding to von Willebrand factor (VWF). *In vitro* binding studies with purified An53, commercial FVIII products, and VWF demonstrated 5-fold greater binding of An53 to human VWF compared to an equivalent B domain-deleted FVIII product. *In vivo* pharmacokinetic studies also appeared to indicate a longer circulating half-life, albeit with lower initial recovery, compared to recombinant human FVIII. Another pharmacologically intriguing aspect of An53 is its decreased reactivity with FVIII inhibitory antibodies. To expand on our initial characterization, we analyzed An53 immune reactivity using a microarray panel consisting of 15 non-overlapping well-characterized anti-human FVIII murine monoclonal antibodies spanning all domains of FVIII. An53 displayed reduced binding to monoclonal antibodies spanning all domains of FVIII. Collectively, these data show that An53 may be an ideal transgene product to investigate in clinical gene therapy for hemophilia A complicated by FVIII inhibitors. Currently, two promising gene therapy approaches for hemophilia A in clinical testing and/or late-preclinical development are *ex vivo* lentiviral vector (LV) gene transfer into hematopoietic stem and progenitor cells (HSPCs) and *in vivo*, liver-directed, adeno-associated viral (AAV) vector delivery. To investigate the potential for An53 in the latter, its cDNA was inserted into a liver-directed AAV cassette and delivered to hemophilia A mice at a limiting dose of 2e11 vector genomes/kg. Mice receiving this vector dose displayed plasma FVIII levels in the range of 100% of the normal human level (1 IU/ml) over the 31 week follow-up. Similarly, when delivered to murine HSPCs at the limiting MOI of 7.4 via a self-inactivating LV encoding An53 driven by a CD68 promoter, and subsequently transplanted into hemophilia A mice, plasma FVIII activity ranged between 0.37 - 1.01 IU/ml during the 16 week follow-up. In both LV and AAV approaches, all mice remained free from inhibitory antibodies directed against the FVIII protein over the course of the study. Collectively, our current findings support An53 as a transgene candidate for clinical development and, more generally, support ASR as an approach to transgene bioengineering.

AAV Vectors I

91. AAV6 K531 Serves a Dual Function in Selective Receptor and Antibody ADK6 Recognition

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Adeno-associated viruses (AAVs) are viral vectors being utilized in several clinical trials for the treatment of genetic disorders with an AAV serotype 1 (AAV1) vector packing the lipoprotein lipase gene being the only approved therapeutic product. However, success of the system is limited by preexisting immunity which prevents gene expression because of antibody neutralization of delivered vectors. Efforts aimed at engineering vectors with the ability to evade the immune response include identification of residues on the virus capsid important for these interactions and eliminating them. Here the specific residue determining monoclonal antibody ADK6 recognition by AAV6, and not the closely related AAV1, was elucidated by structure determination of the AAV6-ADK6 complex by cryo-electron microscopy and image reconstruction followed by molecular biology. The complex structure, determined to ~16Å resolution, identified an ADK6 footprint that extends from the base of the protrusions surrounding the 3-fold axes to the 2/5-fold wall between the depressions at the 2- and 5-fold axes. Molecular biology, biochemical, and cell-based assays identified K531, 1 of 6 out of 736 residues differing between the two viruses, as being responsible for the ADK6 selectivity for AAV6. This residue was previously reported as the determinant of AAV6 binding to heparin and liver tropism which is not shared by AAV1 containing E531. The ADK6 footprint overlaps previously identified AAV antigenic regions. This study thus expands the available repertoire of AAV-antibody information which can guide the design of host immune escaping AAV vectors able to maintain capsid functionality.

92. Tracking Adeno-Associated Virus (AAV) Capsid Evolution by High-Throughput Sequencing

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While recombinant adeno-associated viral (rAAV) vectors show promise, there are still limitations related to their use for gene transfer/therapy. Some of these limitations include pre-existing neutralizing antibodies, low transduction in specific targets, and post-administration immunological responses. In order to increase the repertoire of rAAV vectors with different properties, different molecular strategies have been used to alter the AAV capsid. Our strategy involves making viral DNA libraries after DNA shuffling of different parental AAV capsids and selection schemes in order to obtain rAAV capsids with new infection/transduction properties. However, the optimal selection schemes are not well understood or established. Important parameters affecting the outcome include the capsid library multiplicity of infection (MOI), time of infection, and the number of rounds of selection. Therefore, we set out to track and understand rAAV capsid evolution in a replicating system using high-throughput sequencing. To do this, we developed a highly complex rAAV capsid library (approximately 5×10^6) by shuffling of 10 parental capsids placed into a wild-type AAV2 genome. Importantly, we inserted two sets of barcodes after the capsid polyadenylation site to track capsid diversity by high-throughput sequencing. Our selection process consists of co-infecting the rAAV library with wild-type adenovirus-5 (helper virus), which allows the AAV genomes to replicate in the cells. Our first study involved comparing two library MOIs (10,000 and 250). Our early data showed that while an MOI of 10,000 did not result in high levels of AAV replication, the same selected capsid variant was enriched in three replicates after two rounds of selection in a human hepatoma cell line. In contrast, the use of a lower MOI (250) resulted in high levels of AAV replication, but none of the selected capsid variants

were independently identified in 3 replicate screens after two rounds of selection. Furthermore, we found that the most prevalent selected AAV variants were not necessarily the most efficient vector as measured by transduction of the cell type used for the selection. Moreover, we found that certain capsid variants that are robust for transduction were drastically reduced or even lost with additional rounds of selection. These data suggest that the most logical selection parameters may not be the most robust for selecting capsids with the optimal transduction properties. The barcoded AAV capsid libraries will provide new insights into optimal AAV selection schemes and perhaps provide more robust and clinically useful rAAV vectors.

93. Biology of Capsid and Assembly-Activating Protein of Reptile Adeno-Associated Viruses Isolated from Royal Python and Bearded Dragon

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Adeno-associated virus (AAV) vectors transduce target organs efficiently by direct in vivo administration; therefore, they have been used in various applications for human gene therapy. Although promising, currently available AAV vectors still have issues that need to be resolved for more successful clinical outcomes. One of the important issues in the AAV vector-mediated gene therapy is that human is the natural host of many AAV serotypes and pre-existing immunity against AAVs is prevalent in human populations, which has precluded broader applications of this potentially promising approach to treat various human disease. AAV is a group of viruses that belong to the Dependoparvovirus genus in the Parvoviridae family, and has been found in a wide range of host animal species including mammals, marsupials, birds and reptiles. In the course of seeking AAV strains that might show less prevalent in humans, two reptile AAVs have caught our attention that have been recently isolated from royal python (Snake AAV) and bearded dragon (Dragon AAV). They are new AAV isolates and therefore their biological features remain uncharacterized. To begin to understand the biology of Snake and Dragon AAVs and vectorize them into potential gene delivery vectors, we investigated: 1) whether Snake and Dragon AAV capsid could package the AAV serotype 2 (AAV2) genome in HEK 293 cells; 2) whether capsid assembly requires the assembly activating protein (AAP); 3) whether reptile AAV AAP proteins can also promote assembly of primate AAV strains, AAV1 to 12; and 4) whether the vectors based on these two strains can infect mammalian culture cells. To this end, we constructed a set of plasmid vectors that express Snake or Dragon VP protein, Snake or Dragon AAP protein or AAV2 Rep proteins separately, used them in various combinations together with an AAV-GFP vector plasmid containing the two AAV2 inverted terminal repeats to produce GFP vectors. As a result, we found that both reptile AAV capsids require their cognate AAP for capsid assembly and only Snake AAV can packages AAV2 viral genomes into the capsid with AAV2 Rep in HEK 293 cells. Snake AAP and Dragon AAP are phylogenetically close neighbors but their biological properties are found very different in that, although Dragon AAP can promoter assembly of heterologous capsids including those of AAV8 and 9, the assembly promoting function of Snake AAP is very specific to Snake AAV capsid with no promiscuity. Such a high specificity has never been found in any other AAPs we have studied so far (i.e., AAPs derived from AAV1 to 12, Dragon AAP). A preliminary study using Snake AAV-CMV-GFP vector showed no infectivity to HEK 293 cells. Further studies are warranted to elucidate similarities and dissimilarities of the virus biology between the common AAVs isolated from warm-blooded animals and those from cold-blooded species and to help develop novel AAV vectors.

94. Characterization of Novel Porcine and Caprine Adeno-Associated Virus Capsids for *In Vitro* and *In Vivo* Gene Delivery

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Adeno-associated virus (AAV) has become the leading viral vector used in gene delivery to treat numerous genetic disorders. However, commonly used human AAV serotypes, including AAV2, have high levels of pre-existing immunity in the general population, which can have a detrimental impact on the success of gene therapy. One strategy to circumvent this issue involves pseudotyping AAV vectors with non-human capsids isolated from animal tissue samples. Porcine AAV capsids have demonstrated great promise for the transduction of various tissue types with comparable efficacy to AAV8. We have isolated and characterized two novel AAV capsids of non-human origin, one from porcine colon tissue, AAV.po.Guelph, and the other from a caprine adenovirus stock, AAV.ca.Guelph. Phylogenetic analysis revealed multiple recombination events took place to produce AAV.po.Guelph as various regions are homologous with AAV2, AAV3, AAV7 and other porcine AAV isolates. Conversely, AAV.ca.Guelph is highly similar to bovine AAV without only two amino acids changes, I124L and R499G, and appears unrelated to caprine AAV despite its isolation from goat adenovirus stocks. AAV.ca.Guelph and AAV.po.Guelph were compared to AAV6 in a variety of cell lines including, HEK293, Vero, Caco-2, HepG2, AML-12, rodent lung epithelial cells (RLE) and human fibrosarcoma cells (HTX) (Figure 1). AAV.po.Guelph demonstrated comparable transduction efficiencies as AAV6 in both liver and lung cells lines. Optimization of purification protocols using iodixanol gradients revealed AAV.ca.Guelph was predominantly cell-associated while AAV.po.Guelph was more evenly distributed within the cell pellet and the supernatant. Ongoing mouse experiments will elucidate the *in vivo* transduction profile of these novel capsids following intranasal and systemic delivery of alkaline phosphatase expressing AAV vectors. However based on *in vitro* results we predict that AAV.po.Guelph will have similar transduction efficiency compared to AAV6 in terms of lung and liver transduction.

95. rAAV Is Extensively and Differentially Post-Translationally Modified in Human versus Insect Cell Line Production Methods

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Despite encouraging outcomes in pre-clinical and early rAAV clinical trials, vector potency in several recent trials were discordant. The causative factors underlying this modest performance remain unknown. Different rAAV manufacturing and purification approaches may have contributed to these observations. In addition to potential process impurities, capsid virions may exhibit different chemical characteristics related to the production platform. Thus, we hypothesized that the two leading rAAV manufacturing platforms, baculovirus-infected *Sf9* insect and transiently transfected human HEK-293, result in different capsid post-translational modifications (PTMs). To investigate this, we utilized multiple analytical approaches including deep proteomic profiling with high-resolution and high-mass-accuracy mass spectrometry (nLC-ESI-MS/MS), two-dimensional isoelectric focusing in combination with enzymatic removal of modifications, electron microscopy, structural modeling, and *in vivo* functional analyses. Using a custom dual-use transfer vector plasmid that functions in both insect and human production systems, as well as near identical purification protocols for both production platforms, we found that rAAVs produced in the human and insect cell systems are chemically distinct. We demonstrated that rAAV capsids are both extensively and differentially post-translationally modified in human and insect cell preparations; they are heavily glycosylated, acetylated, phosphorylated, methylated and ubiquitylated. These findings were reproducible across human and insect-produced vectors from numerous rAAV vendors including commercial producers, leading academic core facilities across the U.S., and individual lab preparations. Collectively, these PTMs may have profound implications for capsid folding, viral replication, receptor binding, intracellular trafficking, expression kinetics, functional activity, stability, half-life regulation, immunogenicity, and more. Our findings may inform future directions for resource investments in GMP manufacturing facilities currently being assembled. Clearly, more detailed biochemical and mechanistic investigations are needed to understand the functional role PTMs play in rAAV vector biology. **Key findings that will be presented include:** (1) All rAAV capsid serotypes tested to date are heavily post-translationally modified including: N-glycosylation, acetylation, phosphorylation, methylation and ubiquitylation. (2) rAAV capsids have different PTMs when produced in human versus insect cell systems. (3) Capsids from wtAAV and rAAV productions have different PTMs. (4) PTM density is greater on insect-produced rather than human-produced rAAV capsids. (5) rAAV capsids purified from media supernatant have different PTMs than those from cell lysates. (6) Different serotypes have both highly conserved PTMs across serotypes but also unique PTMs. (7) Sexually dimorphic rAAV expression is seen with vectors produced in human and insect cell systems. (8) Functional studies directly comparing rAAV efficacy from insect and human production methods.

96. A Designer AAV Variant Permits Efficient Retrograde Access to Projection Neurons

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Efficient retrograde access to projection neurons for the delivery of sensors and effectors constitutes an important and enabling capability for neural circuit dissection. Such an approach would also be useful for gene therapy, including the treatment of neurodegenerative disorders characterized by pathological spread through functionally connected and highly distributed networks. Viral vectors, in particular, are powerful gene delivery vehicles for the nervous system, but all available tools suffer from inefficient retrograde transport or limited clinical potential. To address this need, we applied *in vivo* directed evolution to engineer potent retrograde functionality into the capsid of adeno-associated virus (AAV), a vector that has shown promise in neuroscience research and the clinic. A newly evolved variant, rAAV2-retro, mediates up to two orders of magnitude enhancement in retrograde transport compared to commonly used AAV serotypes and matches the efficacy of synthetic retrograde tracers in many circuits. We demonstrate the utility of rAAV2-retro for delivery of effectors including CRISPR/Cas9 to projection neurons. Injection of rAAV2-retro-SaCas9-anti-tdTomato into the basal pontine nuclei of mice expressing tdTomato in cortical layer V excitatory neurons resulted in suppression of tdTomato in 88.6% of infected layer V neurons. Moreover, we show that rAAV2-retro can be combined with Cre driver mice to selectively distinguish between two parallel corticostriatal pathways. In summary, rAAV2-retro permits robust retrograde access to projection neurons with efficiency comparable to classical synthetic retrograde tracers and enables sufficient sensor/effector expression for functional circuit interrogation and *in vivo* genome editing in targeted neuronal populations.

97. Alteration of AAV Capsid Lumenal Residues Expands Genome Packaging Capacity

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Adeno-associated virus (AAV) is widely regarded as the safest viral vector for gene therapy. Moreover, the array of AAV serotypes available allows transduction of different tissues in vivo. However, one major limitation of AAV is the 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 and arginine residues at lumenally exposed sites within the capsid. The additional cationic charge density at the surface may allow for interaction and condensation of vector DNA, which, we believe, leads to an increase in packaging capacity and/or stabilizing the capsid in an over-packaged state. We present in vitro characterization of a library of capsid variants. Variants with intermediate charge (+4 to +7 over wildtype) demonstrate complete encapsidation of vectors up to 5.4 kb in length by alkaline Southern blot. In contrast, wild type AAV-DJ can completely encapsidate 4.7 kb and produces smears, indicative of partial packaging, at longer vector lengths. To validate that these new vectors are functional, we establish that a subset of these vectors are able to transduce mouse liver in vivo. We are currently establishing the relative transduction efficiencies using standard length and expanded genome sizes in order to establish the maximal vector length that can be functionally packaged by these new vectors. Lastly, we have extrapolated our capsid alteration strategy to AAV8 and find that most of the sites altered, based upon the AAV-DJ results, are still amenable to charge alteration and maintain the increased packaging capacity phenotype. We believe that this strategy can be generally extrapolated to other serotypes with distinct tissue tropism and applied in vivo to treat hemophilia A and other monogenic disorders suitable to replacement therapy.

98. Strong Alpha Cell Preference of the AAV Strains That Best Transduce Human Pancreatic Islets In Vitro

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Adeno-associated virus (AAV) vectors offer a powerful tool for the intravenous (IV) delivery of genes to various target tissues in both animals and humans. In order to select the best vector for transduction of human pancreatic islets in vitro, we applied DNA/RNA Barcode-Seq to an AAV library of 40 different AAV strains including common serotypes, several variants, and mutants and performed a comparative assessment of their transduction efficiencies in cultured human islets. This method can determine transduction efficiencies of many AAV strains at the same time in a high-throughput manner by quantifying AAV vector genome RNA transcript levels using Illumina barcode sequencing technology. Our first experiment revealed that AAV2, 3, DJ, LK03, and 2G9 outperform other serotypes and variants in their ability to transduce human islet cells. However, this initial study used a DNA/RNA-barcoded library that used AAV9 as the internal reference control, which was found retrospectively to transduce human islets poorly. To obtain reliable data in the AAV Barcode-Seq analysis, the reference control in a library should transduce the target cells relatively well. Here we show: (1) the result we obtained from the initial study is reproducible over a wide-range of multiplicity of infections (MOIs) when we use a newly prepared, different lot of DNA/RNA-barcoded AAV library with a more appropriate reference control, AAVDJ; and (2) beta cells are much more difficult to transduce than alpha cells with AAVDJ and LK03, which are among the best AAV strains for human islet transduction in vitro. To address data reproducibility, we generated a new AAV library containing 31 different AAV strains with AAVDJ as the internal reference control and used this library to infect human islets at three different MOIs (1.6, 8 and 40 x 10^4) in a triplicated set of experiments. Seven days after infection, total RNA was extracted from the whole islets and subjected to the RNA Barcode-Seq analysis to quantify the abundance of viral genome transcripts of each AAV strain relative to that of the AAVDJ reference control. The result showed that the vector transduction efficiency profiles are MOI-independent, and corroborated our conclusion that AAV2, 3, DJ, LK03 and 2G9 are the AAV strains that transduce human islets in vitro most efficiently. We also compared transduction efficiencies of the 31 different AAV strains in alpha cells and beta cells separately that were FACS-sorted with alpha and beta cell type-specific markers. This experiment demonstrated that the spectra of transduction efficiencies with the best five AAV strains are quite similar between alpha and beta cells. To address a potential difference in the susceptibility to transduction with AAV vectors between alpha and beta cells, AAVDJ and AAVLK03 were vectorized with a CMV-GFP or -TdTomato transgene and used to determine transduction efficiency in alpha cells, beta cells, and other islet cells following exposure of the whole islets to the AAV vector. A flow cytometric analysis revealed that there is a significant difference in transduction efficiency between the cell types, showing a strong preference of alpha cell (77%) over beta cell (9%) or other cells (25%) for AAVDJ (Note: the percentage represents transduction efficiency at an MOI of 4 x 10^5). Similarly, AAVLK03 showed the same trend. These observations highlight the challenge in transducing human beta cells and underscore the importance of further exploring AAV capsids that can transduce human islets with higher efficiency.

99. Haploid Adeno-Associated Virus Vectors Enhance Transduction and Escape Neutralizing Antibodies

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Adeno-associated virus (AAV) vectors have been successfully utilized in clinical trials in patients with hemophilia and blindness. Although the application of AAV vectors has proven safe and is shown to have therapeutic effects in these clinical trials, one of the major challenges is its low infectivity that requires a relatively large amount of virus genomes. Additionally, a large portion of the population has neutralizing antibodies (Nabs) against AAVs in the blood and other bodily fluids. The presence of Nabs poses another major challenge for broader AAV applications in future clinical trials. Effective strategies to enhance AAV transduction and escape neutralizing antibody activity are in high demand. Previous studies have shown the compatibility of capsids from AAV serotypes and recognition sites of AAV Nab located on different capsid subunits of one virion. In this study, we explore whether haploid AAV viruses produced from co-transfection of different AAV helper plasmids have the ability for enhanced AAV transduction and escape of Nabs. We co-transfected AAV2 and AAV8 helper plasmids at different ratios (3:1, 1:1 and 1:3) to assemble haploid capsids. The haploid virus yield was similar to the parental ones, suggesting that these two AAV capsids were compatible. In Huh7 and C2C12 cell lines, the transduction efficiency of AAV8 was much lower than those from AAV2, however, the transduction from all haploid vectors was higher than that from AAV8. The transduction efficiency and the heparin sulfate binding ability for haploid vectors were positively correlated with the amount of integrated AAV2 capsid. After muscular injection, all of the haploid viruses induced higher transduction than parental AAV vectors (2- to 9-fold over AAV2) with the highest of these being the haploid vector AAV2/8 3:1. After systemic administration, 4-fold higher transduction in the liver was observed with haploid vector AAV2/8 1:3 than that with AAV8 alone. Furthermore, we packaged the therapeutic factor IX cassette into haploid vector AAV2/8 1:3 capsids and injected them into FIX knockout mice via the tail vein. Higher FIX expression and improved phenotypic correction were achieved with haploid vector AAV2/8 1:3 virus vector compared to that of AAV8. Strikingly, haploid virus AAV2/8 1:3 was able to escape AAV2 neutralization and had very low Nab cross-reactivity with AAV2. Nevertheless, AAV8 neutralizing antibody can inhibit haploid vector AAV2/8 transduction with the same efficiency as AAV8. Next, we produced haploid vector AAV2/8/9 by co-transfecting AAV2, AAV8 and AAV9 helper plasmids at the ratio of 1:1:1. After systemic administration, 2-fold higher transduction in the liver was observed with haploid vector AAV2/8/9 than that with AAV8. Neutralizing antibody analysis demonstrated that AAV2/8/9 vector was able to escape neutralizing antibody activity from mouse sera immunized with parental serotypes, in contrast to AAV2/8 haploid vector. The results indicate that haploid virus might potentially acquire advantages from parental serotypes for enhancement of transduction and evasion of Nab recognition. This strategy should be explored in future clinical trials in patients with positive neutralizing antibodies.

100. Improved ELISpot Controls to Optimize the Predictive Power of a Novel Canine Disease Model in Gene Therapy for DMD

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A specific iteration of a general problem in gene therapy is the potential for immune recognition of recombinant dystrophin in patients with Duchenne Muscular Dystrophy (DMD), as most cases are caused by multi-exon frame shifting deletions within the dystrophin gene. In contrast, the genetic basis of a widely used large animal model, Golden Retriever Muscular Dystrophy (GRMD), is a single base intron splice mutation that leaves the entire dystrophin coding sequence intact. Muscle from GRMD dogs has been found to express readily detectable amounts of near-full-length dystrophin on the basis of alternative exon splicing. This complicates the interpretation of preclinical studies in this model, as pre-existing tolerance to dystrophin may facilitate long term persistence of transgene products without the risk of autoimmune myositis. Early developmental expression of the dystrophin paralog utrophin in the thymus may confer central immunological tolerance against the peptide sequence. Here we address the immunogenicity of a synthetic version of utrophin (μUtrophin) in which the rod domain has been internally deleted with an emphasis on preserving inter-repeat folding while minimizing neo-epitopes that can trigger T-cell responses. Neonatal GRMD dogs were given an intravenous injection of 10^13.5 vg/kg AAV9-μUtrophin without immunosupression. Peripheral blood mononuclear cells (PBMCs) were collected at 5 and 8 weeks post-injection. An interferon gamma ELISpot assay using utrophin-derived peptide pools revealed the absence of T-cell mediated reactivity, consistent with central immunological tolerance. Our initial positive control for this experiment was injection with Adenovirus-CMV-lacZ, which showed a positive response to lacZ peptide pools. To broaden our study of cell-mediated immunity in the dog, we generated a second positive control by synthesizing a mini peptide pool composed of sequences derived from capsid proteins for canine distemper virus and parvovirus. To our surprise, PBMCs isolated from pre-vaccinated, asymptomatic 6 week-old wild-type dogs resulted in strongly positive gamma-interferon responses to these control antigens, while still negative when tested against utrophin-derived peptides. Further studies are underway to track the potential expansion in reactive T cells post-vaccination in these dogs. These reagents will serve as standard positive controls in future pre-clinical gene transfer studies that explore immunological responses following expression of recombinant test proteins in canine disease models. We outline a rigorous translational approach using the recently characterized deletional-null GSHPMD canine model (VanBelzen, et al, Mol Ther in press). The GSHPMD model should be devoid of any immunological tolerance against all dystrophin-derived peptide epitopes and can therefore provide the most sensitive prediction of immune responses against recombinant dystrophin and/or utrophin expression.

101. A Novel Adeno-Associated Viral Variant Shows Superior Retinal Gene Delivery in Non-Human Primates and Human Retinal Cell Models

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Introduction Gene therapy is potentially a powerful approach to the treatment of inherited and complex retinal disorders. 4D Molecular Therapeutics has applied its Therapeutic Vector Evolution platform to discover novel adeno-associated virus (AAV) variants with superior gene delivery to retinal cells following intravitreal (IVT) administration, a route of administration with significant advantages over other methods of gene delivery to the human eye. Discovery and characterization of variants were performed in non-human primates (NHP). Owing to cross-species differences in AAV gene expression profiles and limitations of animal disease models, retinal pigment epithelium (RPE) and photoreceptors (PRs) were also generated from human pluripotent stem cells (PSCs) to evaluate transduction and tropism of our novel retinal AAV vectors. **Methods** The Therapeutic Vector Evolution platform involves applying progressively more stringent selective pressures to a genetically diverse library of AAV capsids to select novel variants with improved gene delivery over naturally-occurring AAV serotypes. In order to select variants with improved transduction in the primate retina, 4DMT's library of approximately 100 million AAV capsid variants was delivered via IVT injection, variants were isolated from the retinal cells, and the process was repeated until five vector family "hits" were identified. 4D-R100, one of the lead vectors, and AAV2 (control), both carrying a green fluorescent protein (eGFP) reporter cassette, were then delivered by IVT and transduction of retinal cells *in vivo* was assessed by fundus fluorescence imaging. In addition, human RPE cultures were generated from hPSCs using a 45-day differentiation protocol and expressed mature RPE markers including RPE65 and BEST1, synthesized VEGF and PEDF, and phagocytosed rod outer segments. PRs were generated by a multi-step eye cup formation paradigm and expressed Recoverin and S Opsin after 179 days in culture. **Results** We identified novel variants after six rounds of selection. IVT delivery of 4D-R100 carrying a eGFP reporter cassette (4D-R100-eGFP) to NHPs resulted in broader and more robust transgene expression than AAV2 as measured by fluorescence fundus imaging. In Human RPE cultures, 4D-R100-eGFP demonstrated significantly higher transduction efficiency and transgene expression levels seven days post-infection as determined by flow cytometry (2.7-fold increase) and Western blot analysis relative to AAV2. Robust transduction was also observed in the context of PR-directed gene transfer by 4D-R100-eGFP 32 days post-infection. **Conclusions** This study in human retinal cell models as well as NHPs illustrates superior gene delivery by the 4D-R100 variant, discovered through Therapeutic Vector Evolution, as compared to the clinically relevant AAV2. In the future, hPSC-dependent disease models can be used to demonstrate functional rescue, thereby facilitating the path from proof-of-concept to clinical application.

102. Generation of a Shut-Off System for Adeno-Associated Viral Gene Transfer Vectors

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Systems to regulate gene expression from Adeno-associated viral (AAV) vectors are widely used. In most cases, the transgene expression has to be switched on by applying a drug. In terms of safety of gene therapy, a shut-off system for AAV vectors would be beneficial to silence gene expression in case of side-effects, ideally by destruction of the vector. Therefore, we aimed to develop a system for elimination of gene expression from a AAV vectors using an inducible Cre/loxP system that allows tamoxifen-mediated excision of DNA fragments flanked by loxP sites. We generated AAV9 vectors consisting of the inducible $CreER^{T2}$ recombinase, a luciferase reporter gene, and loxP sites inserted at different positions within the vector genome. Four weeks after intravenous vector injection of 10^{12}vg into adult mice, tamoxifen was administered intraperitoneally (1 mg daily, for 5 days) resulting in an up to 10-fold decrease in luciferase activity in heart samples. We further could show that a second tamoxifen administration for another 5 days led to an up to 26-fold reduction in luciferase levels. However, overall expression levels were reduced by insertion of loxP sites (between 2.4 and 5.4-fold). Taken together, delivery of an inducible $CreER^{T2}$ allows efficient inactivation of AAV-mediated gene expression on the expense of reduced overall expression levels due to insertion of loxP sites. Our results contribute to the generation of a novel shut-off system for AAV-mediated gene transfer applicable for the use of various promoters and serotypes.

103. RNA-Seq Tag Counting: An Approach for Quantifying the Relative Expression of Vector-Derived and Endogenous Genes in rAAV-Treated Non-Clinical Models

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Massively parallel transcriptome analysis methods like RNA-Seq are becoming increasingly standardized, prompting their consideration as tools for the analysis of clinical specimens from subjects receiving genetic therapies. Although some regard RNA-Seq to be less quantitative than the more commonly used gene specific qRT-PCR, both methods rely on polymerase based amplification of short DNA amplicons and the efficiency of that process is strongly influenced by the nature of the sequence being amplified. We therefore hypothesized that selective mining of RNA-Seq datasets for carefully controlled amplicon sequence targets might allow robust quantitation of key

individual transcripts with assay characteristics suitable for clinical use. To implement this RNA-Seq Tag Counting (RTC) method, we designed sequence tags that could be used for exact match searching of primary RNA-Seq data files based on the ability of those tags to discriminate between different, relevant alleles of the genes in question, and to have amplification performance characteristics that minimize amplification bias. Tabulation of read count frequencies for the sequence tags (RTC count) in each sample is then interpreted as an indicator of starting RNA quantity. We spiked known quantities of an *in vitro* transcribed RNA molecule matching the expected sequence for vector-derived mRNA into replicate RNA preparations derived from tissues of untreated NHP to establish the sensitivity and linearity of the method. We observed that RTC count is positively correlated to the starting concentrations of the *in vitro* transcribed RNA in a highly linear relationship (R^2 = 0.995) spanning more than two logs and covering the expression range of vector-derived mRNA in non-clinical models. We then selected a subset of transcripts to test the relationship between the coefficient of variation (CV) and the absolute value of the RTC count, which was found to closely resemble the prediction of Poisson distribution, indicating sampling variation as the major source of variation and that RTC method is applicable to most genes. We further established the lower quantification limit as 25 RTC counts, which produces an average of 20% CV, and the lower detection limit as 2 RTC counts when used for distinguishing pairs of transcripts with a single point mutation. The qualified RTC method was implemented to quantify the expression of vector-derived canine myotubularin (MTM1) transcript relative to the endogenous transcript which bears a missense (p.N155K) disease-causing mutation in dog models for X-linked myotubular myopathy (XLMTM) treated with gene therapy. We found that the relative RTC count correlates well with both dose and therapeutic efficacy. Finally, we have implemented the data analysis pipeline for the RTC method on a compliant and secure server platform with the intent to qualify the assay for transcript quantification of human clinical specimens in support of regulatory applications.

104. rAAV-Mediated Combined Gene Transfer and Overexpression of TGF-β and SOX9 Promotes the Chondrogenic Differentiation and Metabolic Activities in Human Bone Marrow Aspirates

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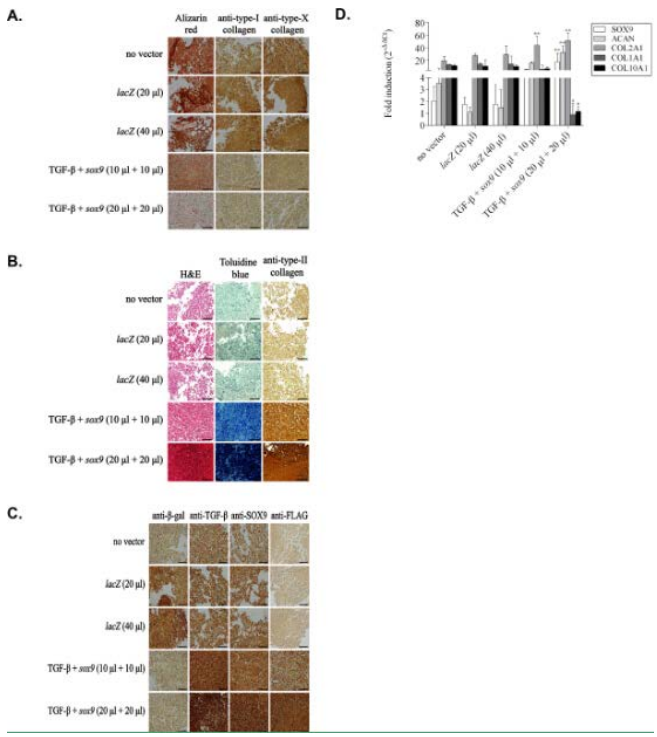
Introduction: Transplantation of genetically modified bone marrow concentrates is an attractive approach to activate the chondrogenic differentiation processes as a means to improve the intrinsic repair capacities of damaged articular cartilage. Here, we examined the potential benefits of co-overexpressing the pleiotropic transformation growth factor beta (TGF-β) and the cartilage-specific transcription factor *sox9* using recombinant adeno-associated virus (rAAV) vectors upon the chondroreparative processes in human bone marrow aspirates. *Methods:* rAAV were packaged, purified, and titrated as previously described. Human bone marrow aspirates were aliquoted in 96-well plates (100 µl of aspirate/well) and immediately transduced with the rAAV vectors (rAAV-*lacZ*: 20 or 40 µl) or co-transduced (rAAV-hTGF-β /rAAV-FLAG-h*sox9*: 10 or 20 µl each vector) with each aliquot (8 x 10^5 functional recombinant viral particles, MOI = 10 ± 3). A volume of 60 µl of chondrogenic medium was then added per aspirate. To assess TGF-β secretion, culture supernatant were collected at the denoted time points. Transgene expression, and expression of type-II, -I, and -X collagen were assessed by immunohistochemical analyses. Hematoxylin eosin for cellularity, toluidine blue for matrix proteoglycans, and alizarin red for matrix mineralization were also measured. *Results:* Successful TGF-β/*sox9* combined gene transfer and overexpression via rAAV was achieved in fresh, chondrogenically induced human bone marrow aspirates for up to 21 days (Fig. 1A), the longest time point evaluated, leading to increased proliferation, matrix synthesis, and chondrogenic differentiation relative to control treatments (reporter *lacZ* treatment, absence of vector application), especially when co-applying the candidate vectors at the highest vector doses tested (Fig. 1B, D). Optimal co-administration of TGF-β with *sox9* also advantageously reduced hypertrophic differentiation in the aspirates (Fig. 1C, D). *Discussion:* Our results first indicate that combined TGF-β/*sox9* gene transfer allowed for the sustained expression of SOX9 as previously noted with single rAAV-FLAG-h*sox9* transduction and to a durable production of TGF-β relative to the control conditions, in the range of those achieved when providing rAAV-hTGF-β alone. The present data further show that prolonged, effective co-overexpression of TGF-β and *sox9* led to increased levels of cell proliferation, matrix biosynthesis, and chondrogenic differentiation in the aspirates over time. Equally important, combined TGF-β/*sox9* transduction advantageously delayed premature hypertrophic differentiation in the aspirates versus control treatments, again in good agreement with the known anti-hypertrophic activities of SOX9 that may counterbalance possible hypertrophic effects of TGF-β. *Conclusion:* These findings report the possibility of directly modifying bone marrow aspirates by combined therapeutic gene transfer as a potent and convenient future approach to improve the repair of articular cartilage lesions.



105. AAV Gene Delivery to the Submandibular Salivary Gland for Salivary-Specific Expression of Adiponectin

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The salivary gland can serve as an ideal target tissue for gene therapy, as it possesses the ability to secrete proteins into the circulation as well as into the saliva. Our lab aims to test the effects of salivary hormones on taste perception, necessitating the need for a mouse model that can express secreted proteins solely in the saliva. In particular, we are interested in studying the role of salivary adiponectin (APN), an adipocyte secreted protein known to regulate body weight homeostasis, in taste perception and obesity. We have shown, for the first time, by whole transcriptome RNA-seq of murine taste buds, that receptors for APN (AdipoR1 and Cdh13) are expressed in taste receptor cells. These RNA-seq results were confirmed by immunohistochemical (IHC) staining, highlighting a potential, yet unexplored role for salivary APN in taste receptor cell signaling. Furthermore, we have conducted behavioral experiments on global APN knockout mice where we measure the response of these animals to a particular taste stimuli and compare their response to that of a wild-type mouse. Results of these behavioral experiments indicate that APN knockout animals have an increased sensitivity to intralipid taste stimuli, while responses to other compounds (bitter, sweet, salty, and sour) were unchanged. To further study this phenomenon, we have constructed Adeno-associated viral (AAV) vectors expressing APN, driven by the α-amylase (*AMY1C*) promoter. These vectors also contain micro RNA target sites for liver specific miR122 and skeletal muscle specific miR206, to prevent transgene expression in off target tissues. We plan to administer these vectors to the submandibular salivary gland of APN knockout mice with the goal of restoring salivary APN levels, while leaving APN absent in the circulation. Generation of this salivary specific mouse model will allow us to study the effects of salivary APN on taste perception and in the future, allow us to measure the effect of salivary APN on body weight homeostasis. This AAV vector construct can also be applied to other areas of research, where specific targeting of the salivary gland is required.

106. Accuracy of Cryo Transmission Electron Microscopy (CryoTEM) in Measuring Amounts of Full and Empty Capsids in AAV Vector Preparations, and Monitoring Gene Therapy Vector Quality and Potency

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Background: A combination of reliable analytical methods is required to measure quality attributes of gene therapy vector preparations based on AAV8, to allow product release and accurate clinical dosing. CryoTEM was established to quantify vector genome-containing and empty vector particles in AAV8 gene therapy vector preparations. Aim: To accurately quantify relative amounts of full, vector genome-containing capsid particles in AAV8 vector preparations using CryoTEM, and to determine the significance of this parameter for potency using orthogonal methods. Methods: Vector samples were preserved close to their native state by the sample preparation method for electron microscope imaging by embedding them in amorphous, non-crystalline ice on an inert support by flash-freezing. Image analysis was done using an automated analysis method. CryoTEM was validated for full/empty quantification using vector preparations with adjusted ratios of full and empty particles generated by mixing vector preparations or selectively enrichening vector fractions by ultracentrifugation. Potency was measured in a cell-based in vitro assay and in an in vivo model. Results: Validation using vector preparations with contents of full particles of 40 - 80% resulted in intermediate and inter-assay precision below a relative standard deviation of 1%. Linearity of six independent experiments was achieved with each r^2 higher 0.996%. Using these vector preparations derived from the same vector bulk also showed a correlation of the full-to-empty ratio with the measured potency. Conclusion: CryoTEM is a valuable tool to allow release of a gene therapy vector preparation for therapeutic use as an orthogonal method to the currently used total capsid particle titer or vector genome titer methods for more accurate vector quantification. This method is also superior to other electron microscope based methods, as its sample preparation principle preserves to a great extent the native state of the sample.

107. Increasing rAAV Vector Production Yields by Reducing the Amount of pAAV-Transgene Plasmid

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Triple transfection is the most widely used method for rAAV vector production. The plasmid ratio, pAd: pRep/Cap: pAAV-transgene, has been optimized for the transfection using the one-factor-at-a-time (OFAT) method for both adherent and suspension cells. In addition, we previously reported that optimization of rAAV vector production in HEK293T suspension cells through Design of Experiment (DoE) showed a new set of parameters: a plasmid ratio of 1:5:0.31, total amount of DNA 1.5 μg/ml and cell density of $2.5x10^6$ cells/ml. However, we experienced that production yields of some rAAV vectors were still challenging. During preparation of rAAV vector the transgene protein is inevitably expressed in the rAAV vector producing cells. This transgene expression may affect the production yields of the rAAV vector. Therefore, we postulated that less amount of pAAV-transgene plasmid may be beneficial for the rAAV vector production yields by decreasing the effects of transgene in the rAAV vector producing cells. Here, we report that serially decreasing amounts of pAAV-transgene down to 1/32 yielded comparable amounts of rAAV vector to that of pAAV-eGFP. Interestingly, we noted that the amount of AAV capsid production was inversely correlated with the amount of pAAV-transgene down to 1/256. These data suggest that there is a fine balance between amount of pAAV-transgene plasmid and AAV capsid expression for an optimal amount of rAAV vector production. Further studies to understand the correlation of transgene expression and rAAV vector production yields are in progress.

108. Analysis of Gene Expression, Tissue Tropism, and Safety of Novel AAV Variants in Mice Following Intravenous Administration

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Adeno-associated viruses (AAVs) show great promise as gene therapy vectors, but naturally occurring serotypes have limited tissue tropism. AAV capsid tropism can be altered by chemical modification, peptide insertion into surface-exposed regions, engineering hybrid variants that have properties of the parental capsids, or by directed evolution. We are developing AAV variants that can overcome the limitations associated with restricted tropism or immune response. For that purpose we sought to examine the biodistribution, safety, and gene expression of several engineered AAV variants following intravenous (IV) delivery in mice. We tested several AAV variants - hybrid capsids, variants with short peptide insertions, as well as those identified by directed evolution. A dose of 1E11 vg of these variants-expressing luciferase driven by the ubiquitous CAG promoter, was intravenously injected into male, hairless SKH-1 mice. In vivo live imaging was performed using the IVIS Spectrum at weeks 2, 4, and 6 to assess luciferase expression kinetics. Animals were sacrificed at week 6 and blood, liver, heart, brain, lungs, spleen, pancreas, kidneys, quadriceps, and gonads were collected using ultra-clean procedure. Tissues were analyzed using reverse transcriptase PCR followed by quantitative PCR for levels of luciferase mRNA. Several variants showed improved expression profiles in terms of levels of luciferase expression or more tissue-specific gene expression. Also, most variants exhibited a good safety profile, with luciferase mRNA limited to the tissues of interest. Finally, genomic DNA analysis of blood revealed traces of some vectors at the 6 week time-point. Modification of AAV capsids by peptide insertions in receptor binding regions or novel capsids discovered by directed evolution can lead to variants with desired tropism. We plan to perform studies to assess long-term efficacy of these variants in non-human primates to further their development.

109. Rapid AAV Titer Determination Using NanoSight: Improvements to the Labelling Protocol and Preliminary Results for Fluorescent Genome Labelling

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The development of adeno associated virus (AAV) gene therapies requires rigorous quantitative testing. In particular, an accurate particle titer measurement is important for purification and to determine viral dosimetry. Current methods to determine AAV titer include qPCR, for measuring genome copy, ELISA, for measuring capsid count, and AUC, to measure genome empty/full ratios. We have previously shared a gold labelling protocol that enables the rapid measurement of AAV particle concentration using Nanoparticle Tracking Analysis (NTA). This protocol entails a 30-minute gold labelling period, followed by a five-minute measurement time that provides a high resolution size distribution of gold labelled AAV, total virus count, and aggregation state. Here, we present AAV NTA results after an additional year of development work, and describe the resulting improvements to the gold nanoparticle labelling protocol. These improvements generalize the labelling conditions to expand the compatibility to additional AAV serotypes and vehicle buffer conditions. Also, we present preliminary NTA fluorescent results of AAV genomes using SYBR-Gold. Combined, these approaches have the potential to provide empty/full viral ratios as well.

110. AAV and Adenovirus Purification Made Simple

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Recombinant viral vectors based on adeno-associated virus (AAV) are promising vectors for gene therapy. Adenovirus is a promising oncolytic virus and has been utilized in numerous trials as a gene therapy vector. A challenge commonly associated with the use of these viral vectors is the difficulty and expense involved with small to large scale production and purification. Expensive and highly specialized equipment, such as ultracentrifuges, are required for classical purification methods through a cesium chloride gradient. To greatly decrease the time required and the need for ultracentrifugation, we have utilized a silica carbide resin for rapid purification of AAV and adenovirus across a range of scales

(0.5 mL to 1 liter+ of input) via ion exchange chromatography. Purified AAV and adenovirus was shown to be highly biologically active in in vitro experiments, and in vivo experiments are currently on-going.

Cancer-Immunotherapy, Cancer Vaccines I

111. Gene Modification of Human T Cells via Piggyback Yields an Anti-BCMA CARTyrin Cellular Product with Durable Efficacy

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Genetic modification of primary human T cells has emerged as a powerful tool in developing immunotherapies for multiple diseases. Specifically, chimeric antigen receptor (CAR)-modified T cells are effective in treating acute lymphoblastic leukemia and have shown promise against other malignancies, including multiple myeloma (MM). T cell engineering is typically achieved via transduction, most notably with lentivirus. Despite extensive optimization of these viral vectors, limitations including cost and time for GMP manufacturing, safety concerns regarding the insertional profile, limited cargo capacity, and an undesirable phenotype of the final product still exist. Here, the utility of piggyBac (PB) to efficiently modify human primary T cells is demonstrated. Using PB we have engineered T cells with greater durability and persistence in preclinical xenograft mouse models, likely due to improved product phenotype comprising a high percentage of stem cell memory T cells.

P-BCMA-101 is an autologous T cell product modified to express an anti-BMCA CARTyrin through an electroporation reaction containing a DNA plasmid transposon and mRNA encoding a hyperactive PB transposase. A CARTyrin is a CAR that employs a FN3-based binding moiety that replaces the traditional single chain variable fragment in most CAR constructs. Due to the large cargo capacity of PB, a suicide switch, the CARTyrin, and a mammalian selection cassette are encoded in a single transposon vector. Prior to selection, gene expression is detected in 20-35% of cells within 2 days of transposition. After a single round of activation and selection, a >95% CARTyrin$^+$ T cell population can be harvested and frozen ready for infusion. This method of gene editing results in >7 x 10^9 modified T cells from a single apheresis product without exogenous cytokine supplementation.

Preclinical evaluation of P-BCMA-101 has revealed an improved phenotype and longer *in vivo* persistence compared to lentiviral transduced CAR-T products. The final cell product consistently yields 70-80% stem cell memory cells (CD45RA+, CD62L+, CD95+, CCR7+, CD27+, CD28+, and CD127+). In addition, the final cell product expresses low levels of exhaustion markers including PD1, Tim-3, and LAG3. *In vitro*, P-BCMA-101 cells kill >80% BCMA+ tumor cell lines within 24 hours, as well as demonstrate potent killing of numerous primary patient tumor samples. *In vivo* efficacy was evaluated in a fully disseminated NSG mouse model of human MM using luciferase$^+$ MM.1S cells at dose levels of 4 x 10^6 and 12 x 10^6 cells/mouse while monitoring tumor burden for 92 days. Tumor bearing untreated control mice succumbed to disease within 29 days of P-BCMA-101 dosing, while treated mice saw a reduction in tumor burden to levels below the limit of detection by bioluminescent imaging within 7 days. While tumor relapse did occur around day 28-35 in several mice at both dosing levels, tumor from relapse was subsequently eliminated without additional product dosing. This second response was durable out to the conclusion of the experiment, demonstrating unparalleled durability of P-BCMA-101 *in vivo* with the ability to control tumor relapse.

In conclusion, PB modification of human T cells is efficient, allows for larger cargo delivery, and preferentially transposes stem cell memory cells, leading to increased efficacy, durability of response, and persistence of administered product. Based on this preclinical work, P-BCMA-101 is being moved forward for phase I/II clinical trials.

112. A Novel TGF-β/IL-12R Signal Conversion Platform That Protects CAR T Cells from TGF-β-Mediated Immune Suppression and Concurrently Amplifies Effector Function

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Numerous immune-suppressive mechanisms exist within the tumor microenvironment that may hinder chimeric antigen receptor (CAR) T cell efficacy. 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 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. A dominant negative receptor version (DNR) of TGFBR2 that does not contain signaling domains protects T cells from the impacts of TGF-β by blocking the ability of TGF-β to induce pSMADs. Here, we report the development of a novel TGF-β signal conversion platform that provides a T cell stimulatory 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 the T cell simulating IL-12 receptors IL-12R-β2 and IL-12R-β1, respectively. Using a single lentiviral vector encoding both chimeric TGF-β receptors (CTBR12) and a CAR, we demonstrated that CAR-CTBR12 T cells were completely protected from TGF-β-mediated SMAD phosphorylation. In addition, CAR-CTBR12 T cells generated significant amounts of

pSTAT4 and pSTAT5 in response to TGF-β exposure, a response that mimics the T cell stimulation effects of IL-12. To further demonstrate successful signal conversion, we evaluated the impact of TGF-β exposure on the secretion of IFNγ, a major downstream target of IL-12 signaling. CAR-CTBR12 cells secreted significantly greater amounts of IFNγ than either control CAR T or CAR-DNR T cells following activation in the presence of TGF-β. We next utilized a 3-week serial re-stimulation assay in the presence of exogenous IL-2 to expand CAR, CAR-DNR, and CAR-CTBR12 T cells in the presence or absence of TGF-β. As expected, TGF-β exposure resulted in a significant inhibition of antigen-driven T cell proliferation in control CAR T cells. By contrast, both CAR-DNR and CAR-CTBR12 cells were protected from TGF-β-mediated inhibition of expansion. Gene expression analysis following 21 days of weekly antigen-driven expansion revealed specific TGF-β-mediated gene expression changes in CAR-CTBR12 cells consistent with IL-12 signaling and increased T cell potency, including significant upregulation of IFNG, IL10, IL18RAP, IL18R1, IL21R and CD62L transcripts. Lastly, we evaluated CAR-CTBR12 cells in a stringent 2-week serial re-stimulation assay in the presence of TGF-β but absence of exogenous IL-2 cytokine support. We showed that CAR and CAR-DNR T cells failed to expand and clear tumor cells in the second week of the assay, while CAR-CTBR12 cells completely cleared the second tumor challenge specifically in the presence of TGF-β. Together, these data demonstrate the successful development of a TGF-β signal conversion platform that transforms the inhibitory effects of TGF-β exposure into an IL-12R-like T cell stimulatory signal that has the potential to produce superior CAR T cell responses *in vivo*.

113. Activating CD40 While Inhibiting IL6R Induces Cytokine Production without PDL1 Upregulation in DCs

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A tumor lesion consists of tumor cells surrounded by stroma including fibroblasts, blood vessels, immune cells and extracellular matrix. The tumor microenvironment (TME) supports the progression, metastasis and resistance of tumor cells as well as supporting expansion of regulatory immune cells. In some tumors, such as pancreatic cancer, the TME is dense due to overproduction of collagen and the tumor is heavily infiltrated with different myeloid cells such as M2 macrophages and myeloid-derived suppressor cells. CD40 is an important costimulatory receptor present on myeloid cells but it is also present on epithelial cells, endothelial cells and fibroblasts. Signaling via CD40 ligand (CD40L) drives production of cytokines and chemokines. Another costimulatory receptor, 4-1BB, is expressed by lymphocytes and dendritic cells (DCs). 4-1BB stimulation drives lymphocyte expansion and induce memory cells. IL6 receptor (IL6R)-signaling leads to STAT3 phosphorylation in myeloid cells, which can lead to suppressive phenotypes. Further, STAT3 signaling enhances production of TGF-beta that promotes overexpression of collagens. We have constructed a family of oncolytic adenoviruses called LOAd that activates the CD40 pathway via a trimerized membrane-bound CD40L (TMZ-CD40L). To strengthen the effect of immune activation we have tested such a virus combined with 4-1BBL or a single chain fragment targeting the IL6R. In the present study, the LOAd viruses (-, 700, 703, 713) were investigated for their capacity to activate human monocyte-derived DCs as well as their effect on pancreatic tumor cells and stroma using flow cytometry, MTS assay and ProSeek Proteomics. Further, the role of CD40-mediated apoptosis was investigated. Dendritic cells increased costimulators, cytokines as well as chemokines upon TMZ-CD40L signaling but PDL1 was not expressed when IL6R was blocked during activation. Infected tumor cells died by oncolysis but prior death they reduced factors such as amphiregulin and Spp1 that otherwise promote tumor progression. CD40 positive tumors were more efficiently killed by TMZ-CD40L-containing LOAd virus in vitro and in immunodeficient mouse models, which was due to an enhanced apoptosis. Infected fibroblast-like stellate cells obtained from human pancreas reduced tumor-promoting factors such as FGF5, PlGF, amphiregulin, Gal3, TGFb and collagen type I. Finally, the LOAd viruses were active across several human tumor xenograft models including tumors of the pancreas, bladder, lung and colon. Taken together, our data demonstrates that it is possible to utilize oncolytic adenoviruses armed with activators or inhibitors of important pathways that regulate immunity and the biology of the stromal cells.

114. UCART22: An Allogenic Adoptive Immunotherapy of Leukemia by Targeting CD22 with CAR T-Cells

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Adoptive immunotherapy using autologous T-cells endowed with chimeric antigen receptors (CARs) has given rise to long-term durable remissions and remarkable objective response rates in patients with refractory leukemia, raising hopes that a wider application of CAR technology may lead to a new paradigm in cancer treatment. However, a limitation of the current autologous approach is that CAR T-cells must be manufactured on a "per patient basis". To overcome this limitation, we have developed a standardized platform for manufacturing T-cells from third-party healthy donors to generate allogeneic "off-the-shelf" engineered CAR$^+$ T-cell-based frozen products. Our allogenic platform utilizes the Transcription Activator-Like Effector Nuclease (TALEN$^®$) gene editing technology to inactivate the TCRα constant (*TRAC*) gene, significantly reducing the potential for T-cells bearing alloreactive TCR's to mediate Graft-versus-Host Disease (GvHD). We have previously demonstrated the precise and efficient disruption of the *TRAC* gene by gene editing, yielding up to 85% of TCRαβ-negative cells, and allowing efficient production of TCRαβ-deficient T-cells that no longer mediate alloreactivity in a xeno-GvHD mouse model. In the clinic, the proof of concept of the applicability of our allogeneic platform was achieved with early compassionate use for patients treated with UCART19, an allogeneic engineered CAR T-cells product directed against CD19. UCART19 clinical trials are currently ongoing. Here, we have developed T-cells targeting CD22 which is expressed

on tumor cells from the majority of patients with B-cells leukemia. In a first step, we have screened multiple antigen recognition domains in the context of different CAR architectures to identify effective CAR candidates displaying activity against cells expressing variable levels of the CD22 antigen. As a safety feature, T-cells are engineered to co-express a depletion gene, rendering them sensitive to the monoclonal antibody rituximab. Several constructs of depletion genes have been evaluated in the context of the CD22 CAR. In addition, experiments in an orthotopic ALL mouse model using UCART22 cells demonstrated important anti-tumor activity *in vivo*. The ability to carry out large scale manufacturing of allogeneic, non-alloreactive CD22 specific T-cells from a single healthy donor can offer the possibility of an off-the-shelf treatment that would be immediately available for administration to a large number of leukemic patients. UCART22 could also offer an alternative to patients who may relapse with CD19-negative tumors after CD19 CAR T-cell treatment.

115. Lymphocyte-Nanoparticle Biohybrids as a Combined Nanoimmunotherapy for Cancer

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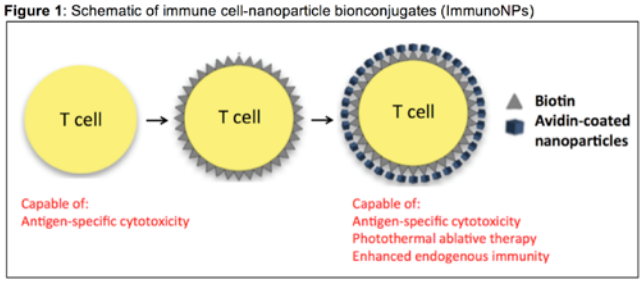
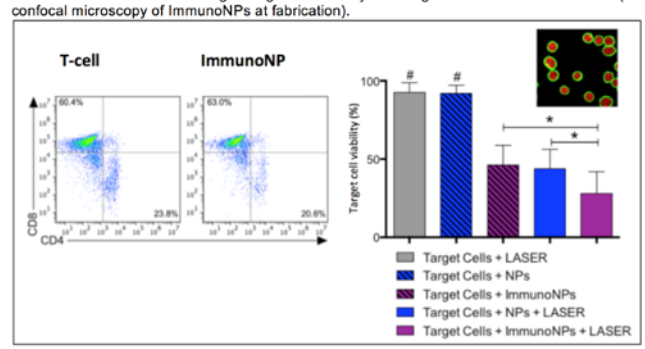
T cell therapies have shown promise against leukemias, but little efficacy against solid tumors. Success is limited by an immunosuppressive tumor environment, which precludes effector cell accumulation at the tumor site or renders effector cells dysfunctional thus preventing tumor clearance. As such, strategies to improve effector cell function at the tumor site have the potential to enhance clinical responses. We have observed that multifunctional nanoparticles can confer additional properties to existing cell-based immunotherapies including ablative heating, magnetic responsiveness, and localized drug delivery. We thus sought to evaluate whether immune cell-nanoparticle biohybrids (ImmunoNPs, **Fig.1**) could combine the potent cytotoxic capabilities of antigen-specific T cells and ablative therapy from nanoparticles to enhance immune responses within the suppressive tumor microenvironment.

Figure 1: Schematic of immune cell-nanoparticle bionconjugates (ImmunoNPs)



We synthesized a robust biohybrid capable of antigen-dependent cytotoxicity, followed by localized ablative therapy to efficiently eliminate residual disease by conjugating T-cells with Prussian blue nanoparticles (which absorb light in the near infrared range). We demonstrated T stable cell-nanoparticle conjugation over at least 3 days (51-65.8% by flow cytometry). T-cells within the biohybrid retained their proliferative ability (66.4% for T-cells vs. 66.5% for biohybrid by CFSE dissolution) and effector phenotype (mean 62.7% CD8+ T-cells vs. 55.2% CD8+ biohybrid, n=7 **Fig. 2**), with no significant increases in markers of exhaustion (PD1, TIM3, LAG3). Furthermore, we demonstrated improved cytotoxicity against tumor antigen-expressing target cells following treatment with ImmunoNPs: each component individually was able to decrease target cell viability from 92.7% (target cells alone) to 46.3% (T-cells alone) or 43.8% (NPs with laser), however maximal eradication occurred with the tandem biohybrid (target cell viability of 28%, **Fig. 2**). Additionally, we found that ablative therapy with non-cellularized Prussian blue nanoparticles was capable of increasing tumor lymphocyte infiltration 3-fold ($p<0.05$) compared to untreated tumors in vivo, suggesting that photothermal ablation can augment endogenous immune responses.

Figure 2: (Left) Effector phenotype of T-cells alone or in the ImmunoNP biohybrid, and (Right) functional readout of tumor-antigen target cell viability following treatment with ImmunoNPs (inset: confocal microscopy of ImmunoNPs at fabrication).



We believe this work represents a novel modality that combines the strengths of cell-based immunotherapy with nanomedicine in order to achieve maximal therapeutic responses to challenging malignancies and infectious diseases.

116. Propagating Humanized BLT Mice for the Study of Human Immunology and Immunotherapy

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The humanized BLT (bone marrow-liver-thymus) mouse model harbors a nearly complete human immune system, therefore providing a powerful tool to study human immunology and immunotherapy. However, its application is greatly limited by the restricted supply of human $CD34^+$ hematopoietic stem cells and fetal thymus tissues that are needed to generate these mice. The restriction is especially significant for the study of human immune systems with special genetic traits, such as certain HLA (human leukocyte antigen) haplotypes or monogene deficiencies. In order to circumvent this critical limitation, we have developed a method to quickly propagate established BLT mice. Through secondary transfer of bone marrow cells and human

thymus implants from BLT mice into NSG (NOD/SCID/IL-2Rγ$^{-/-}$) recipient mice, we were able to expand one primary BLT mouse into a colony of 4-5 proBLT (propagated BLT) mice in 6-8 weeks. These proBLT mice reconstituted human immune cells, including T cells, at levels comparable to that of their primary BLT donor mouse. They also faithfully inherited the human immune cell genetic traits from their donor BLT mouse, such as the HLA-A2 haplotype that is of special interest for studying HLA-A2 restricted human T cell immunotherapies. Moreover, an EGFP reporter gene engineered into the human immune system was stably passed from BLT to proBLT mice, making proBLT mice suitable for studying human immune cell gene therapy. This method provides an opportunity to overcome a critical hurdle to utilizing the BLT humanized mouse model and enables its more widespread use as a valuable pre-clinical research tool.

117. Novel Anti-CD30 Chimeric Antigen Receptors with Fully-Human Variable Regions Function Better with a CD28 Domain Than with a 4-1BB Domain

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Although most patients with Hodgkin lymphoma can be cured with first-line therapies, 15-27% of patients will progress to relapsed or refractory lymphoma indicating a need for improved treatments. Chimeric antigen receptor (CAR) T-cell therapies have powerful activity against B-cell malignancies by targeting CD19 expressed on the surface of malignant cells. Hodgkin lymphoma and T-cell lymphomas do not express CD19, but many cases of Hodgkin lymphoma and T-cell lymphoma uniformly express CD30. We have therefore constructed a series of CARs that contains the single chain variable fragment (scFv) from a fully human anti-CD30 antibody. Use of a CAR containing variable regions from a fully-human antibody should reduce recipient anti-CAR immune responses. One CAR, designated Hu30-CD28z, includes hinge, transmembrane, and the cytoplasmic portions of the CD28 co-stimulatory molecule. The second CAR, designated Hu30-CD828z, includes hinge and transmembrane regions from CD8-alpha and a CD28 costimulatory domain. The third CAR, designated Hu30-CD8BBz, is identical to the second CAR except the CD28 domain is replaced with a 4-1BB costimulatory domain. All three CARs include the cytoplasmic portion of the CD3-zeta T-cell activation molecule. When T cells were transduced with a lentiviral vector containing the CAR genes, all 3 anti-CD30 CARs were consistently expressed at high levels on transduced T cells. Although T cells expressing each of the anti-CD30 CARs demonstrated CD30-specific activity including degranulation, cytokine release, and proliferation, we found that T cells expressing Hu30-CD28z produced higher levels of IFNγ and TNF-α than Hu30-CD828z or Hu30-CD8BBz. T cells expressing Hu30-CD8BBz showed the lowest level of cytokine release in response to CD30$^+$ target cells but also the highest level of non-specific activation when either cultured alone or with CD30-negative target cells. Soluble CD30 protein neither blocked CAR T-cell recognition of CD30$^+$ target cells nor lead to nonspecific CAR T cell activation when added to cultures containing CAR T cells alone. We evaluated the ability of T cells expressing each construct to eliminate tumors in vivo by establishing tumors in mice using CD30$^+$ HH cells. With this model, we found that T cells expressing the Hu30-CD28z CAR were able to completely eliminate tumors with 100% of mice cured at doses ranging from 2x10^6 to 8x10^6 T cells per mouse. T cells expressing Hu30-CD828Z were also effective at eradicating tumors and curing mice. Interestingly, T cells expressing Hu30-CD8BBz were not able to eliminate tumors at the 2x10^6 T cells/mouse dose level despite in vitro activity against CD30$^+$ targets. Because T cells expressing Hu30-CD28z showed high levels of specific activity against CD30$^+$ targets in vitro and in vivo, this CAR has been chosen for further evaluation in a clinical trial.

118. Comparative Efficacy of CD19 Chimeric Antigen Receptor T Cells Expressed Using Gamma-Retroviral Vector or Lentiviral Vector

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Clinical trials in patients with advanced B cell leukemias and lymphomas treated with CD19-specific chimeric antigen receptor (CAR) T cells have induced remissions in adults and children. However, the duration of response and persistence of the CAR-modified T cells in the trials has varied. Here we have tested the hypothesis that the choice of vector might contribute to the differential persistence of the CAR T cells. A CD19 CAR (CAR19) used in clinical trials was expressed using a lentiviral vector or a γ-retroviral vector. In vitro experiments showed no difference in cellular phenotype, target cell killing or cytokine production from these CAR T cells. However, in a murine model of acute lymphoblastic leukemia, significantly enhanced tumor clearance and survival was observed with the lentiviral-modified CAR19 T cells compared to γ-retroviral-modified CAR19 T cells. Mechanistic studies indicate that the EF-1α promoter in the lentiviral vector was more efficient than the LTR promoter in the γ-retroviral vector, and that there was a more homogenous distribution in levels of surface expression in lentiviral CAR T cells. Further, when stimulated by leukemia cells that express CD19, the growth of lentiviral CAR19 T cells was superior to γ-retroviral-modified CAR T cells. In summary, our studies indicate that lentiviral vectors are superior for the eradication of leukemia in xenograft models, in marked contrast to in vitro assays that show similar efficacy of CAR T cells using γ-retroviral and lentiviral vector technology. These results indicate that the choice of vector and its promoter are critical factors in the efficacy of CAR T cells, and may explain the more persistent expression of lentiviral CAR T cells in clinical trials.

119. Optimizing the CAR Spacer to Improve T Cell Potency

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Chimeric antigen receptor (CAR) T cell therapy has recently emerged as an attractive approach for the treatment of hematological malignancies. However extending the success of this strategy to other targets has proven to be more complicated that simply replacing the scFv. To address this issue we have implemented a form of adaptive CAR design whereby a series of sequential modifications were made to the CAR spacer region and subsequently tested in vitro and in vivo. We illustrate the utility of such a strategy using our second generation CAR.PSCA (v1.0), which contains an IgG1-CH2CH3 spacer. To i) minimize Fc-FcγR interactions and ii) attenuate tonic signaling we constructed a panel of vectors with the following modifications to the spacer region: 1) mutated IgG1 to decrease Fc-FcγR interaction (v2.0); 2) substituted mutated IgG1 framework to IgG2 (v3.0); 3) deleted both CH2 and CH3 from IgG2 framework (v4.0); and 4) included the CH3 region from IgG2 framework (v5.0). We now demonstrate how modifications made to this single CAR structural domain resulted in enhanced (i) T cell migration, (ii) antigen recognition, and (iii) cell phenotype, ultimately producing superior anti-tumor effects. First, by abrogating Fc-FcγR interactions with CAR v2.0 and v3.0 we were able to improve T cell migration, which was evident in NSG mice engrafted s.c. with Capan-1 and treated i.v. with FFluc+ T cells. Ten days post CAR administration we saw a 2 log increase in the T cell signal at the tumor site ($4.5\pm2.3x10^5$ p/s vs $4.8\pm0.5x10^7$ p/s vs $4.0\pm1.1x10^7$ p/s, CAR v1.0, v2.0 and v3.0 respectively). Subsequently, due to decreased tonic signaling, T cells modified with CAR v4.0 exhibited a less differentiated T cell phenotype (T_{naive}: 1.8±0.6% to 19.2±4.0%, T_{CM}: 10.4±1.4% to 14.1±3.0%, T_{EM}: 83.5±1.2% to 53.7±6.9% and T_{EMRA}: 4.3±0.9% to 12.9±1.7% - CAR v3.0 and CAR v4.0, respectively), which enhanced in vivo persistence. Indeed, when administered to Capan-1-engrafted NSG mice CAR v4.0 T cells exhibited enhanced in vivo longevity as measured using bioluminescence imaging ($7.3\pm4.6x10^7$ p/s CAR v3.0 vs $2.8\pm1.74x10^8$ p/s CAR v4.0 T cells - day 35 post-administration). Finally, antigen recognition of CAR.PSCA was improved in v5.0 by re-incorporating the CH3 region, resulting in superior anti-tumor effects against a PSCA-dim target tumor cell line (DU145) in a 6hr ^{51}Cr-release assay (20.7±5.8% vs 48.4±5.2%, CAR v4.0 vs CAR v5.0, 40:1 E:T). Overall, implementation of this adaptive design produced a CAR T cell product with enhanced in vivo anti-tumor activity. This was clearly illustrated when we compared the tumor volume of NSG mice treated with CAR v1.0 or CAR v5.0 T cells (1309±143 mm^3 vs 510±53 mm^3 on Day 66).

120. Adenovirus-Based Epitope Vaccine for Cancer Immunotherapy

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Background: Immunotherapies that precisely target tumor-specific antigens (TSAs) promise improved cancer treatment outcomes, minimal side effects, and reduced treatment costs. The efficacy and safety of immunotherapies depend on the availability of TSAs that are not present in normal tissues and constitute unique targets for therapy. These patient-specific targets, which often differ from their normal homologues by only minor structural changes, offer the best hope for successful eradication of tumors by immunotherapies. TSA epitopes must be targeted very precisely to avoid triggering undesired autoimmunity against the parts of the mutated protein that are identical to its normal counterpart. The synthetic peptides comprising target TSA epitopes that are traditionally used to achieve this pinpoint precision are often poorly immunogenic. **Hypothesis**: Precise targeting of TSA epitopes may be achieved by raising strong and highly specific immune responses through the ordered, multivalent presentation of such epitopes on the surface of highly immunogenic adenovirus (Ad) particles. **Approach and Results**: To test this hypothesis, we have used the Pep3 epitope of the truncated form of epidermal growth factor receptor as a model epitope. Pep3 represents a unique 13-amino acid-long sequence, LEEKKGNYVVTDH, which is formed as a result of aberrant splicing of EGFR cDNA that often happens in tumor cells (*i.e.*, malignant gliomas) and yields a truncated form of receptor, EGFRvIII. To present Pep3 on the surface of Ad virions, we have genetically engrafted the Pep3 in the hypervariable region 5 (HVR5) of the hexon protein of human Ad serotype 5, Ad5. This insertion has been well tolerated by the viral proteins: we have rescued and propagated Pep3-modified virions; neither the virus infectivity, nor the virus yields were affected. By vaccinating C57BL/6 mice with this hexon-modified Ad, we have been able to show Pep3-specific immune responses, both humoral (ELISA, Western blotting) and cell-mediated (ELISpot). In addition, we have engrafted the Pep3 epitope in the knob domain of Ad5 fiber protein - at the carboxy-terminus or in the HI loop. These recombinant knobs have been expressed in E.coli and their native trimeric configuration has been confirmed by SDS-PAGE and size exclusion chromatography. By probing these proteins with a polyclonal anti-Pep3 antibody (ELISA), we have shown dramatic insertion site-dependent difference in epitope recognition efficacy. In summary, we have demonstrated that (1) a short TSA-specific epitope can be genetically engrafted in Ad5 capsid proteins, (2) vaccination of mice with such epitope-engrafted Ads results in humoral and cell-mediated immune responses; and (3) the antigenicity of Pep3 epitope engrafted in Ad capsid proteins depends on the exact location of the engraftment site. These data support the use of Ad5 virions for presentation of tumor-specific epitopes to induce highly specific anti-tumor immune responses in cancer patients.

121. Effect of CAR-T Therapy on Bone Lesions in Myeloma Mouse Model

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Introduction: Multiple myeloma (MM) is an incurable hematological malignancy of plasma cells. During the past decade, overall survival rate of multiple myeloma have been improved. These improvements are linked to the induction of novel drugs with different mechanisms of action such as proteasome inhibitors and immnomodulatory drugs. However, MM remains in most cases an incurable disease in most cases, and new therapeutic strategies are urgently required for radical cure or continued disease control. During recent years, some dramatic responses have been reported using T cells expressing CD19-targeted chimeric antigen receptors (CAR). Therefore, CAR therapy could also be a promising new therapeutic strategy for MM. CD269 (also known as BCMA: B-cell maturation antigen) is a membrane protein that is selectively expressed on B-cell lineages and on plasma cells including myeloma cells. Our goal of this study is to verify whether treatment with CD269-specific CAR-expressing T cells can eradicate myeloma cells from bone marrow of tumor-bearing NOG mice. **Methods:** Firstly, we uniquely developed monoclonal antibodies against human CD269. Next, we designed and verified novel CD269-specific CAR. The CD269-CAR recombinant retroviral vector encodes the MoMLV (Moloney murine leukemia virus) retroviral backbone and the 3.3E-28z CAR. The 3.3E-28z CAR consists of an anti-human CD269 scFv that was derived from the 3.3E mouse hybridoma, a portion of the human CD28 molecule and the intracellular domain of the human CD3ζ molecule. **Results:** CD269-specific CAR-expressing T cells showed redirected cytolysis toward CD269-positive U266 human MM-derived cells, but not CD269-negative K562 cells. Luciferase-expressing U266 cells that were injected into the cardiac chamber of NOG mice, selectively infiltrated to bone marrow. Six weeks after tumor inoculation, we injected saline, non-*CAR* gene-modified T cells, or *CAR* gene-modified T cells (GMCs) into the cardiac chamber of tumor-bearing mice. In the group of GMC injection, U266 cells were dramatically eradicated from bone marrow. Finally, the extent of the bone legions in each model was assessed by micro-CT. Bone lesions in mice injected with U266 cells result in osteolytic bone destruction of the femur that usually accompanies the growth of myeloma cells. However, GMC injection clearly suppressed the progression of bone destruction. **Conclusion:** Although the efficacy of BCMA-CAR has been demonstrated by previous study using intradermal models of MM, our challenge is the first report that presents with orthotopic models of MM. Our results are more appropriate for predicting the efficacy of CD269-CAR in the treatment of MM. We conclude that adoptive transfer of CD269-CAR-expressing T cells could be a promising option for patients with MM.

122. Engineered Macrophages Engorge on Tumors Cells Inhibiting Migration Leading to Accumulation, Shrinkage, and Differentiation

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Marrow-derived macrophages can be highly phagocytic, but whether they can also be made to accumulate in solid tumors and engulf cancer cells is questionable given known limitations of tumor associated macrophages (TAMs). By inhibiting marrow-derived macrophages from recognizing other cells as 'self', tail-vein injected donor cells accumulate in solid tumors in proportion to their engorgement of antibody-opsonized cancer cells. Inhibition of SIRPα on both mouse and human donor macrophages impedes recognition of 'self-marker' CD47 on targeted cells to drive tumor shrinkage for at least 1-2 wks in studies of xenografts and a syngeneic melanoma model. Whereas TAMs are rarely positive for engulfment of opsonized tdTomato tumors and shrink tumors slowly if at all after tumor-CD47 knockdown, nearly all donor macrophages in tumors are strongly positive for engulfment, with phagocytosis and accumulation predicting tumor shrinkage. Although donor macrophages differentiate upon tumor-localization, with SIRPα increasing towards levels on non-phagocytic TAMs, multiple injections of engineered macrophages can nonetheless re-initiate shrinkage of solid tumors *in vivo*. Blood parameters remain normal throughout, suggesting safety as well as efficacy.

123. Mechanism of Action of a Retroviral Replicating Vector, TOCA 511 & 5-FC: Levels of Infection for Anti-Tumor Activity and Immunity

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Toca 511 (vocimagene amiretrorepvec) is an investigational, conditionally lytic, retroviral replicating vector (RRV). The vector selectively infects cancer cells because productive infection is dependent on cell division, and viral replication is enabled in tumors by the immune suppressed microenvironment and by genetic defects in the interferon signaling pathways. Toca 511 infects and spreads through cancer cells and stably delivers the gene for an optimized yeast cytosine deaminase that, upon administration of the prodrug Toca FC (an investigational, extended-release formulation of 5-fluorocytosine), generates 5-fluorouracil (5-FU) within infected cancer cells. In animals, the combined treatment directly kills cancer cells and also immune suppressive myeloid cells in the tumor microenvironment leading to T cell infiltration and activation of antigen presenting cells. In such models, Toca 511 administered intratumorally or intravenously can infect the majority of cancer cells. Data from human trials show that: 1) a significant number of delayed but durable complete and partial responses have been observed after treatment of High Grade Glioma patients with Toca 511 and Toca FC, supporting an immune mechanism; and 2) it is difficult to know what percent of, or total number of, cancer cell infections leads to benefit. These observations raise the question of how much transduction is sufficient to generate a

useful anti-tumor response. We have addressed this issue in an animal model. To avoid the use of exogenous drugs which could distort outcomes, we blocked the infectious spread of Toca 511 in tumors at various defined levels, then evaluated survival and generation of anti-tumor immunity. Initial data suggests that even a small percent of infection shows meaningful benefit.

Cancer-Oncolytic Viruses I

124. Preclinical Study of Tumor-Targeted and Armed Oncolytic Vaccinia Virus for Systemic Cancer Virotherapy

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Vaccinia virus, once widely used for smallpox vaccine, has been engineered and used as an oncolytic virus for cancer virotherapy. Epidermal growth factor (EGF)-like vaccinia growth factor (VGF) is a secreted protein produced early in viral infection and contributes to viral spread and replication via EGFR-dependent MAPK/ERK1/2 activation. O1 protein is another activator of the pathway located downstream of the EGFR, complementing the function of VGF. Our study is the first to demonstrate that deletion of both VGF and O1 genes inhibit pathogenic viral replication in normal cells without impairing therapeutic replication in tumor cells, and furthermore engineer the VGF-/O1-VV armed with bifunctional fusion gene expression of cytosine deaminase and uracil phosphoribosyltransferase (CD/UPRT) which converts the prodrug 5-FC into the chemotherapeutic 5-FU and 5-fluoroUMP.

The tumor specificity and oncolytic activity were evaluated by single intraperitoneal or intravenous injection of VGF-/O1-VV in clinically relevant mouse models of peritoneal dissemination or liver metastasis of human pancreatic cancer. The models of peritoneal dissemination were developed by intraperitoneal injection of human pancreatic cancer cell lines (AsPC-1, BxPC-3, MIA PaCa-2, PANC-1, Panc 10.05 and SW1990) which are stably expressing Renilla luciferase. Furthermore, cancer stem cells (CSCs) expressing high level of CD44 v9 were sorted from the AsPC-1 cells. The CSCs were intrasplenically implanted into nude mice for the development of liver metastasis. Noninvasive bioluminescence imaging of viral distribution and tumor growth demonstrated the complete or partial tumor regression following the treatment of VGF-/O1-VV. Importantly, the viral replication of VGF-/O1-VV encoding Firefly luciferase in addition to CD/UPRT was detected in the tumor tissue which showed upregulation of the pERK1/2 levels, but not in normal tissues. Thus, systemic injection of VGF-/O1-VV in mouse models of pancreatic cancer significantly prolonged survival compared with mock therapy, and the combination with 5-FC enhanced its therapeutic index. The MAPK-dependent tumor-specific replication of VGF-/O1-VV was also confirmed in ex vivo infection of live tissues from pancreatic cancer patients.

On the other hand, preclinical toxicology evaluation of VGF-/O1-VV has been being undertaken by intravenous administration into male and female cynomolgus monkeys. Inoculation of VGF-/O1-VV at a dose of 5 x 10e7 pfu/kg was well tolerated and did not lead to any viral shedding, while the lower dose of VGF-/O1-VV fully elicited potent antitumor effects in the above tumor mouse models.

125. Deletion of the C Protein Improves the Oncolytic and Immunotherapeutic Potential of Measles Virus

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Live-attenuated strains of measles virus (MV) are naturally oncolytic against a wide variety of human cancers, whose sensitivity depends on the overexpression of the CD46 receptor at the cell surface and/or defects in the type I interferon (IFN) response pathway upstream or downstream of the IFN receptor IFNAR. Different attempts have been made to improve MV specificity for cancer cells, MV tracking in vivo or MV safety, for instance by inserting the gene of IFN-β in MV genome. We hypothesized that the deletion of the C protein from MV could have positive effects on several aspects of its oncolytic activity. First, MV-ΔC is expected to induce faster tumor cell death, as the C protein has a critical role in delaying apoptosis of infected cells by promoting autophagy. Second, the C protein is also involved in inhibiting the type I IFN response and deletion of this protein would make MV-ΔC safer than unmodified MV, especially in normal cells. Finally, faster cell death induction associated with an increased production of both cellular and viral danger signals would be of particular interest for MV-ΔC to better activate anti-tumor immune responses. When used in vitro against human malignant mesothelioma (MM) cells, MV-ΔC induced faster cell death than parental MV in previously shown MV-sensitive cells, despite a lower replication efficacy of MV-ΔC due to the absence of the C protein. These enhanced oncolytic properties were confirmed in vivo in immunodeficient mouse models in which a single injection of the virus was sufficient for rapid regression of orthotopically-grown peritoneal MM xenografts. As we hypothesized, MV-ΔC-related tumor cell death was associated with increased quantities of HMGB1 and ATP danger signal release than what was observed with unmodified Schwarz MV. To test whether this promoted activation of human immune cells, MV-ΔC-infected tumor cells were co-cultured with human dendritic cells (DCs), which showed enhanced activation with increased expression of maturation markers such as CD83. As we previously showed how oncolytic MV could efficiently prime T cells through activated myeloid and plasmacytoid DCs, our current experiments aim at understanding what makes MV-ΔC more immunogenic. We also focus on studying these immune mechanisms in vivo in new immunocompetent murine models that will help to evaluate both direct anti-tumor activity and immunotherapeutic

properties of oncolytic strains of MV against aggressive malignancies. These findings will help to design future oncolytic viruses such as MV with improved immuno-oncolytic activity.

126. Enhanced Antitumor Efficacy of Interleukin-12-Expressing Oncolytic Herpes Simplex Virus Type 1 Combined with Induced Pluripotent Stem (iPS) Cell-Derived Dendritic Cells in Bladder Cancer

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Introduction: Oncolytic herpes simplex viruses type 1 (HSV-1) that are genetically engineered to replicate selectively in neoplastic cells are promising new therapeutic agents for solid tumors. A phase I clinical trial for patients with prostate cancer using G47Δ, a third-generation oncolytic HSV-1, is ongoing in Japan. On the other hand, dendritic cell (DC) vaccine is an effective tool of cancer immunotherapy, although the necessity to obtain individual DCs from each patient has been a big hurdle for its clinical application. Recent advances in the induced pluripotent stem (iPS) cell technology provides a useful source for functional DCs. This study investigates the antitumor efficacy of the combination of oncolytic virus therapy with iPS cell-derived DCs (iPSDCs), using a syngeneic murine bladder cancer model. ***Methods:*** We used T-mfIL12, a triple-mutated oncolytic HSV-1 with the G47Δ backbone armed with murine interleukin-12. iPSDCs were derived from a murine embryonic fibroblast-derived iPS cell line, and their functional analyses were comprehensively performed (morphology; expression of surface molecules; antigen uptake assay; mixed lymphocyte reaction; and popliteal lymph node assay). C57BL/6 mice bearing subcutaneous MB49 bladder tumors were sequentially treated with intratumoral T-mfIL12 (on days 0 and 5) and either iPSDCs or conventional bone marrow-derived DCs (BMDCs) (on day 2), and the tumor volume was monitored. Systemic antitumor immune responses were evaluated by interferon-γ ELISpot assay using splenocytes harvested on day 15. ***Results:*** Comprehensive evaluations confirmed that iPSDCs had sufficient DC-like features in terms of both morphology and function. A significantly better tumor growth suppression was observed in the T-mfIL12 + iPSDCs group compared with the T-mfIL12 alone group, which was comparable to that in the T-mfIL12 + BMDCs group. ELISpot assay demonstrated equivalent increases in interferon-γ expression in these groups. ***Conclusions:*** T-mfIL12 in combination with iPSDCs was shown to exhibit an enhanced antitumor efficacy equivalent to that with BMDCs in a syngeneic murine bladder cancer model. Various potentials of iPSDCs considered, this combined viro-immunotherapy might be a potent treatment strategy suited for clinical application.

127. Modification of Neurovirulent Herpes Simplex Virus Type1 Strain by Means of Natural Selection Through Human Glioma Xenografts of Neurovirulent Herpes Simplex Virus Type1 Strain by Means of Natural Selection Through Human Glioma Xenografts

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Oncolytic Viruses (OVs) are genetically modified to be attenuated to minimize toxicity to normal tissue and to retarget their toxicity to tumor cells. However, since host-virus interactions at the molecular level are highly complex and context-dependent, the a priori genetic modification of OVs remains empirical. Natural selection may be another approach to create more effective viruses for tumor therapy. In this study we utilized a wild-type HSV1 strain harvested from a human patient with HSV1 encephalitis and we repeatedly passaged this virus in nude mice with human glioblastoma xenografts. After 10 rounds of serial passage, we propagated and purified the passaged virus for subsequent experiments. In order to analyze the difference between parent and passaged virus, we checked replication capability and anti-tumor efficacy using glioma cells in vitro and in vivo. We also checked the distribution of antigen presenting cells in response to virus infection. There was a statistical significant increase in the replication rate of the passaged virus compared to the parent virus and there was also a trend for enhanced anticancer efficacy of the passaged virus compared to the parental virus. Furthermore, we observed a significant decrease of CD45+ lymphocytes infiltrates in glioma bearing brains infected with passaged virus compared to parent virus. We conclude that natural selection of viruses by serial passage in vivo environment may provide tumor-adaptive changes that may be beneficial for anticancer therapy.

128. Deletion of Adenovral Death Protein from the Adenoviral E3 Region Improves Imaging Potential of Oncolytic Virus Therapy

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Previously we have reported on oncolytic adenoviruses (OAd) modified to contain imaging or therapeutic genes such as eGFP, interferon, and sodium iodine symporter (NIS) for tumor diagnosis and therapy. In this vector structure, the transgene was placed in the adenoviral E3 region, while enhanced oncolysis was mediated by overexpression of adenoviral death protein (ADP). Although this structure was operative in detection and therapeutic regimens for local and metastatic tumors, we were concerned that the cytolytic effect of ADP may affect NIS membrane localization. The improper localization of NIS can lead to suboptimal conditions for radioisotope uptake thus hampering its potential as a well-needed tumor diagnostic and monitoring tool. We therefore designed identical ADP-deleted OAds (ADP-) and

assessed the impact of ADP on radio-isotope uptake and imaging in pancreatic cancer. Western blot and immunocytochemical analyses of pancreatic cancer cells infected *in vitro* with either wild-type or Cox2-promoter controlled ADP(-) demonstrated higher NIS expression when compared to the ADP(+) counterpart. This was correlated with an improved radioiodine (^{123}I) uptake. Next, we performed a series of SPECT-CT imaging studies in a mouse model to visualize human Panc1 and Patient Derived Xenografts through the monitoring of technetium ($^{99}mTcO^{4-}$). A single injection of OAdCoxADP(-) accumulated significantly greater amounts of $^{99}mTcO^{4-}$ when compared to ADP(+). The ADP(-) produced a strong non-invasive signal until day 32 outlasting that of ADP(+) and replication-deficient AdCMV-NIS vectors. Immunohistochemistry revealed a significantly higher NIS tumor expression with ADP(-) than with ADP(+). Importantly, ADP(-) showed a distinct NIS cell membrane pattern as it co-localized with membrane bound Cytokeratin 4. In contrast, ADP(+) vectors showed a punctate NIS tissue-staining pattern, with little to no cell membrane localization. These results support our hypothesis that the cytolytic effect from ADP-overexpression disturbs the cell membrane integrity, subsequently affecting NIS localization and radiotracer uptake. Surprisingly, although ADP(+) alone produced the greatest tumor suppressive effect, the tumor shrinkage and Ad-hexon levels with ADP(-) treatment were comparable to that with ADP(+) and were significantly better than with AdCMV-NIS. To further evaluate the clinical potential of OAdCox2ADP(-), we assessed the therapeutic effect in combination with Iodine-131 (I^{-131}) in mice with human pancreatic cancer xenografts. OAdCox2ADP(-) in combination with I^{-131} greatly slowed tumor progression greater than OAd or I^{-131} alone. The detection of I^{-131} with a gamma counter showed a clear trend where ADP(-) retained higher I^{-131} in tumor tissues than ADP(+). These findings support the clinical applicability of ADP-deleted OAds as more sensitive tools for NIS-based cancer diagnosis and therapy. We are currently investigating the effect of ADP on other imaging and/or therapeutic genes expressed from the OAd E3 region.

129. Prodrug Activator Gene Therapy Using Retroviral Replicating Vectors in an Experimental Model of Human Osteosarcoma

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Retroviral replicating vectors (RRVs) have been shown to achieve efficient tumor transduction and enhanced therapeutic benefit in a wide variety of cancer models. Here we evaluated two different RRVs derived from amphotropic murine leukemia virus (AMLV) and gibbon ape leukemia virus (GALV), which utilize different cellular receptors for viral entry, in human osteosarcoma cells.

We analyzed 1) expression levels of the cellular receptors for GALV (PiT-1) and AMLV (PiT-2) by quantitative RT-PCR, 2) Viral replication, and 3) RRV-mediated prodrug activator gene therapy in human osteosarcoma cell lines, as well as normal cells.

Both receptors (for PiT-1 and PiT-2) were expressed in most osteosarcoma cell lines and normal cells tested in this study. However, RRVs expressing the green fluorescent protein gene efficiently infected and replicated in most osteosarcoma cell lines, but not in normal cells. Among the osteosarcoma cell lines, low PiT-1 but high PiT-2 expression was observed in HOS, MG-63 and Saos-2 cells, and AMLV but not GALV spread efficiently in these cells in culture. Furthermore, RRVs expressing the cytosine deaminase prodrug activator gene showed differential cytotoxicity that correlated with the results of viral spread. AMLV-RRV-mediated prodrug activator gene therapy achieved significant inhibition of subcutaneous MG-63 tumor growth in nude mice.

These data indicate the potential utility of AMLV-RRV-mediated prodrug activator gene therapy in the treatment of human osteosarcoma. Furthermore, multiple RRVs that utilize different cellular receptors for entry may be highly useful for RRV-mediated prodrug activator gene therapy against different types of solid tumors, by enabling customization of virotherapy on the basis of cellular receptor expression profiles.

130. Intratumoral Delivery of Oncolytic Adenovirus Encoding Decorin and Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) Evoked Anti-Tumor Responses via Growth Inhibition, Metastasis Blockade and Immune Activation

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Colorectal cancer (CRC) is the fourth most common cause of cancer related deaths worldwide. In advanced and metastatic stages of CRC, reduced sensitivity to conventional strategies is a major obstacle to overcome. Decorin, a prototypic member of the small leucine-rich proteoglycans (SLRPs), is an pivotal regulator in the development and progression of various cancers. Previous results from our group and others demonstrated that decorin is significantly down-regulated in tumor lesions of CRC patients and related with the tumor metastasis and recurrence. In this study, we developed rAd.DCN.GM, an oncolytic adenovirus encoding decorin and GM-CSF. Firstly, we showed that rAd.DCN.GM mediated the decorin and GM-CSF expression effectively, and produced cytotoxicity in both human and murine CRC cell lines, including SW480, SW620 and CT26. We found that cell lysates from rAd.DCN.GM infected SW480 cells could stimulate the proliferation of peripheral blood mononuclear cells (PBMCs) from CRC patients, and enhanced the expression of Th1 cytokines, Granzyme B and perforin. To investigate the anti-tumor effects of rAd.DCN.GM, CT26 xenograft model was established in immune competent BALB/c mouse subcutaneously. rAd.DCN.GM was administrated intratumorally on

day 7 (2.5×10^{10}VPs), and a repeat injection was conducted on day 10. We showed that rAd.DCN.GM not only inhibited the growth of local tumors, but also prevented the tumor metastasis to the lungs. Our mechanistic studies showed that all of the oncolytic adenoviruses induced apoptosis and inhibited the proliferation of tumor cells at tumor sites. In the tumor sites, rAd.DCN.GM produced decorin expression effectively and secreted into sera. rAd.DCN.GM-mediated decorin also down-regulated angiogenesis and epithelial mesenchymal transition (EMT) markers, such as VEGF, N-cadherin and Vimentin. Furthermore, rAd.DCN.GM could further activate viral and tumor directed immune responses. rAd.DCN.GM treatment rapidly increased the population of $CD8^+$ T lymphocytes on day 12, and slightly up-regulated $CD4^+$ T lymphocytes on day 29, suggesting that GM-CSF might activate $CD4^+$ T memory cells. Importantly, rAd.DCN.GM also inhibited TGF-β expression and promoted the proliferation and maturation of DCs in the spleen. In conclusion, an oncolytic adenovirus rAd.DCN.GM treatment inhibited the growth and metastasis of colon cancer, via down-regulating target genes of decorin and activating the anti-tumor immune responses. These results suggest that rAd.DCN.GM is a potential novel oncolytic vector that can be developed as an effective anti-tumor agent in the clinic. (Y.Y. and Z.L. made equal contributions)

131. Targeting an Oncolytic Adenovirus to Cancer Cells Using the Chemokine Ligand CXCL12

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Breast cancer is the most commonly diagnosed cancer in women under 60. Treatment of localized breast cancer results in a high survival rate; however, the survival rates for advanced disease are poor. Thus, standard therapies are inadequate in treating advanced stage disease. As the limits of existing treatment regimens for breast cancer are recognized, novel therapies clearly necessary for the successful treatment of carcinoma of the breast. Oncolytic adenoviruses are a promising therapeutic tool to enable the virus-mediated lysis of infected tumor cells. Attributes such as large DNA incorporation capacity, ease of genetic manipulation, high gene transfer efficiency, systemic stability, and low pathogenicity in humans make the adenovirus a suitable vector for a variety of oncolytic virotherapy applications. Despite being attractive delivery vehicles, applying serotype 5 adenovirus vectors in clinical trials has been limited by their poor infection efficiency in cells that express low levels of the native hCAR (the human coxsackievirus and adenovirus receptor). Adenovirus infection is initiated by the binding of its fiber knob domain to the hCAR in host cells. Since hCAR is downregulated in many cancers, development of novel strategies to enhance viral affinity toward tumor cells over normal cells is a key approach to overcome a major hurdle in cancer therapy application. Therefore, we have directed our efforts to retarget the adenovirus vector toward CXCR4, a seven-membrane spanning G-protein-coupled receptor, whose overexpression is implicated in a wide variety of metastatic tumors, including breast cancer. Recently, we retargeted a replication-deficient adenovirus construct using a recombinant bispecific adapter protein containing the soluble extracellular domain of the hCAR fused to the mature human chemokine ligand for CXCR4 through a short peptide linker. CXCL12 (also known as SDF-1) is a CXC chemokine that is widely expressed in a variety of tissue types and functions as a potent chemoattractant for immature and mature hematopoietic cells. In the current study, we engineered a replication-competent oncolytic adenovirus, in which the CXCL12 ligand replaced the fiber knob domain. To achieve novel specificity of oncolytic adenovirus infection of cancer cells that overexpress CXCR4, we inserted the CXCL12 sequence into the fiber gene. We created a recombinant fiber gene containing 80 amino acids from the N-terminus of the Ad fiber (corresponding to the tail domain), 257 amino acids from the bacteriophage T4 fibritin protein (containing the fibritin shaft and foldon trimerization domains), a 15 amino acid spacer (GGGGSGGGGSGGGGS), and 68 amino acids from the the mature human chemokine CXCL12/SDF-1a protein. The resulting vector was rescued and amplified in HEK293 cells for subsequent characterization. Initially, we will test virus specificity of binding to the CXCR4 receptor as well as cell killing efficiency in a panel of breast cancer cells. These studies will test the hypothesis that retargeting of an oncolytic adenovirus using the CXCL12 would allow selective infection and killing of CXCR4 expressing cancer cells, and provide a strong rationale for developing retargeted adenovirus therapies against metastatic disease.

132. Mild Hyperthermia Induced by Gold Nanorod-Mediated Plasmonic Photothermal Therapy Enhances Transduction and Replication of Oncolytic Adenoviral Gene Delivery

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Oncolytic adenovirus (Ad) is a promising candidate for cancer gene therapy. However, as a monotherapy, it has shown insufficient therapeutic efficacy in clinical trials. In this work, we demonstrate that gold nanorod (GNR)-mediated mild hyperthermia enhances the cellular uptake and consequent gene expression of oncolytic Ad to head and neck tumor cells. We examined the combination of oncolytic Ad expressing vascular endothelial growth factor promoter-targeted artificial transcriptional repressor zinc-finger protein and GNR-mediated mild hyperthermia to improve antitumor effects. The in vitro mechanisms of increased transduction in the presence and absence of hyperthermia were explored followed by evaluation of efficacy of this combination strategy in an animal model. Exposure to optimized hyperthermia conditions improvedendocytosis of oncolytic Ad, transgene expression, viral replication, and subsequent cytolysis of head and neck cancer cells. GNR-mediated plasmonic photothermal therapy resulted in precise control of tumor temperature and induction of mild hyperthermia. A combination of oncolytic Ad and GNRs resulted in potent tumor growth inhibition of head and neck tumors.

133. The Risk of Hepatocellular Carcinoma from AAV Gene Therapy

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Adeno-associated viruses (AAVs) are a promising vector for use in liver targeted human gene therapy. However, previous murine studies have revealed that introduction of AAVs into neonates can result in the formation of hepatocellular carcinomas (HCC). Part of the AAV vector can integrate and cause the overexpression of a specific location on chromosome 12 that spans multiple genes including Rian and Mirg. As this region is conserved in the human genome, it raises a concern for the safety of AAV in human gene therapy trials. To assess the risk that AAVs may cause HCC in human patients, a human hepatocyte chimeric mouse model was used. Firstly, to establish that a human mouse chimeric HCC model can be generated, human hepatocytes were transduced ex-vivo with AAV vectors expressing the oncogenic SV40 large T antigen or HRAS prior to being transplanted into immune compromised, Fah knockout (FRGN) mice. To assess the risk of AAV integration causing HCC, a similar repopulation experiment where the CMV enhancer/β-actin promoter was inserted into the MEG8 locus (the human homolog of the mouse Rian gene) by homologous recombination with the use of CRISPR/Cas9 genome editing technology was done. These mice were subsequently monitored for HCC through the formation of tumors. We are also using a similar AAV dual vector strategy in non-human primates. Neonatal and adult Rhesus macaques will be infected with AAVs containing Cas9 and a targeting vector comprising of a guide RNA and a CMV enhancer/β-actin promoter targeted to the Rhesus macaque MEG8, CCNA2 or TERT loci. These animals will be monitored for the formation of HCC. Results from these studies will aid in the assessment of the safety of AAV as a vector for gene therapy treatments and potentially establish new models for the study of human HCC.

Cancer-Targeted Gene and Cell Therapy I

134. A Single CRISPR-Mediated Knockout as Novel Suicide Switch and Selection Tool for Gene-Modified T Cells

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Successful clinical use of genetically modified T cells requires a balance between therapeutic potency and patient safety. The application of novel strategies to target malignancies through chimeric antigen receptors (CARs) and genetic enhancements to improve T cell expansion and persistence, pose unpredictable risks to patients, would become safer if endowed with a safety switch. Several problems limit the use of current "suicide systems", for example, the immunogenicity of the transgene (HSV-TK), the bioavailability of antibodies and the limited availability of inducing agents (AP1903 for iCaspase9).

To determine if knockout of the hypoxanthine phosphoribosyltransferase 1 (HPRT) gene could be used as a suicide strategy in CAR-T-cells, we applied our recently-established, highly efficient CRISPR/Cas9 protocol for gene disruption. The HPRT enzyme facilitates the recycling of purines into purine nucleotides. Hence, T cells lacking HPRT are solely dependent on the *de novo* purine biosynthesis pathway for survival. A bonus of HPRT knockout is that HPRT negative (HPRT-) cells can also be enriched by addition of the toxic nucleoside analog 6-Thioguanine (6-TG) to cell culture, which eliminates HPRT expressing (HPRT+) cells that incorporate 6-TG via the purine salvage pathway.

After 6-TG selection, we blocked the *de novo* purine synthesis in CD3-stimulated CD19.CAR T cells for 72 hours *in vitro* using therapeutically relevant doses of mycophenolic acid (MPA) (1-100 uM), we specifically eliminated over 95% of the HPRT– CAR T cells while HPRT expressing (HPRT+) T cells remained affected (Figure 1).

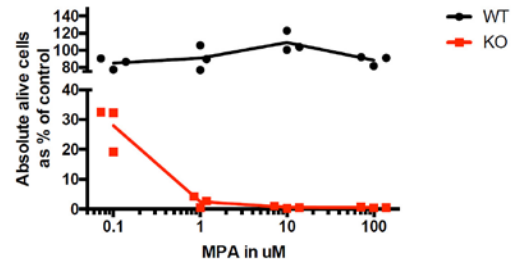

Figure 1
CD3-stimulated CAR T cells were cultured for 72 hours in purine-containing medium with IL-2 (100 units/ml) and mycophenolic acid (MPA). The absolute number of vital cells per well was normalized to the vehicle-treated control to adjust for donor variability. (N=3)

HPRT- CD19.CAR T cells proliferated on average 25% less than their wildtype counterparts in purine-containing medium with IL-2 (100 units/ml), but they retained their ability to kill CD19+ tumor cells and to secrete cytokines in response to CAR stimulation.

Finally, in the presence of up to 100uM of 6-TG, HPRT- CD19.CAR T cells continued to exhibit proliferative function and cytotoxic activity in serial co-culture while HPRT+ CAR T cells were eliminated by the drug. This highlights the chemoresistant properties of HPRT- CAR-T cells and suggests they could be used in combination with 6TG for the treatment of many 6TG-sensitive cancers.

In summary, we present a transgene-free safety solution that ensures on one hand the controlled depletion of T cells with clinically available drug, MPA, and on the other hand induces 6-TG resistance enabling positive selection *in vitro* and potentially synergistic anti-tumor efficacy of chemotherapy and CAR T cells in patients. We are currently evaluating the suicide strategy in xenograft mouse models.

135. A Dual-Switch Platform to Orthogonally Control CAR-T Efficacy and Safety with Two Non-Immunosuppressive Chemical Inducers of Protein Dimerization

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Background: Chimeric Antigen Receptor (CAR) strategies are effective as T cell therapy against disseminated cancers. To improve their efficacy against solid tumors, a platform was devised to separate tumor antigen-specific first generation CARs from a cytosolic costimulatory component, iRMC, triggered by a non-immunosuppressive analog of rapamycin, BPC007. To de-risk off-tumor reactivity or excessive cytokine release, a suicide switch, iC9, was included providing rapid, rimiducid-dependent CAR-T cell apoptosis.

Results: A novel non-immunosupressive analog of rapamycin was generated. The added bulk of the chemical 'bump' on BPC007 reduced affinity and hence inhibition of mTOR/TORC1 but retained subnanomolar affinity for a mutant FKBP-Rapamycin Binding (FRB) domain, KLW. KLW was fused in tandem with wild-type FKBP and the costimulatory signaling domains of MyD88 and CD40 to create iRMC. Following BPC007 administration, NF-κB activity was stimulated in a robust and dose-dependent fashion (EC_{50} < 1 nM). When incorporated into a dual-switch retroviral vector (iRMC-2A-iC9-2A-CAR) and incubated with antigen-specific tumor cells, BPC007 addition stimulated T cell proliferation, cytokine production and dose-dependent tumor cell killing. In 7-day coculture assays, BPC007-treated HER2-specific CAR-T cells effectively eliminated SKBR3 breast carcinoma cells (E:T, 1:1), SKOV3 ovarian carcinoma (E:T, 1:5), or HPAC (E:T, 1:15) pancreatic carcinoma cells, whereas non-BPC007-treated cells exhibit poor antitumor efficacy. When rimiducid was included in CAR-T cultures, T cell apoptosis was rapidly induced ($T_{1/2}$ = 6 hours for microscopic observation of fluorescent caspase-3 substrate). Despite the fact that both iRMC and iC9 incorporated FKBP12 domains, the costimulatory and safety switches were orthogonally regulated due to the allele specificity of rimiducid for the F36V variant of FKBP12.

Summary: A unified dual-switch vector system that promotes both inducible costimulation and safety to CAR-T therapy is demonstrated.

136. Preclinical Development of CD7 CAR T Cells for the Treatment of Acute Myeloid Leukemia

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Acute Myeloid Leukemia (AML), the commonest leukemia in adults, has a high mortality rate due to chemoresistance and relapse. CD7 is a transmembrane protein expressed in ~30% of AML with higher chemoresistance and correspondingly poor prognosis. Normal expression of CD7 is confined to T- and NK-cells, limiting potential "off-tumor on-target" toxicity. In this study, we explored the feasibility of targeting AML with T-cells expressing a CD7-specific chimeric antigen receptor (CAR). We created several CD7 CAR constructs using single chain variable fragment (scFv) sequences derived from three CD7-specific mAbs. Due to high levels of residual CD7 expression, expression of all CD7 CARs completely abolished the expansion of transduced T cells. To overcome this limitation, we used CRISPR/Cas9 to disrupt CD7 expression in ~90% of activated T cells with a CD7-specific single guide RNA. CD7 deletion did not inhibit T cell effector functions, and subsequent transduction of these CD7-knockout ($CD7^{KO}$) T cells with CD7 CARs resulted in robust expansion, comparable to that in control activated T cells. $CD7^{KO}$ CD7 CAR T cells demonstrated robust cytotoxicity against CD7+ AML cell lines KG-1a and Kasumi-3, resulting in 80-99.9% elimination of malignant cells after 3 days of co-culture at a 1:4 effector-to-target ratio, regardless of the scFv clone. $CD7^{KO}$ CD7 CAR T cells reduced primary AML cells by 80% after 48 hours of co-culture at a 1:1 effector-to-target ratio. Moreover, there was, on average, a 26-fold reduction in leukemic colony formation following 5-hour coculture of $CD7^{KO}$ CD7 CAR T cells with primary AML cells, suggesting that CAR T cells can recognize and eliminate primitive leukemic progenitors. To assess the reactivity of $CD7^{KO}$ CD7 CAR T cells against normal hematopoietic cells, we cocultured them with cord blood and measured subsequent myeloid colony formation. We saw no difference in the number or size of monocytic, erythrocytic or granulocytic colonies after coculture with control or $CD7^{KO}$ CD7 CAR T cells. We also found $CD7^{KO}$ CD7 CAR T cells were not cytotoxic against peripheral blood monocytes after 24h coculture at a 1:1 effector-to-target ratio. Hence, $CD7^{KO}$ CD7 CAR T cells appear non-toxic to normal myeloid cells. However, $CD7^{KO}$ CD7 CAR T cells do kill normal, CD7-expressing T- and NK-cells. As $CD7^{KO}$ T cells are able to mount a normal response to viral peptides but are resistant to the CD7-directed cytotoxicity, infusing $CD7^{KO}$ T cells may provide immune reconstitution and reduce the risk of immunodeficiency. In summary, we show that genome editing enables generation of functional CD7 CAR T cells that produce robust cytotoxic activity against AML cell lines and primary AML blasts but show no

toxicity against normal myeloid cells and their progenitors. These results demonstrate the potency and support the feasibility of using CD7 CAR T cells for the targeted therapy of acute myeloid leukemia.

137. SOCS-1 Gene Therapy Improves Irradiation-Resistance Through Targeting on the Inhibition of the STAT3 Activation in Esophageal Squamous Cell Carcinoma

Takahito Sugase[1], Tsuyoshi Takahashi[1], Satoshi Serada[2], Minoru Fujimoto[2], Tomoharu Ohkawara[2], Kosuke Hiramatsu[3], Koji Tanaka[1], Yasuhiro Miyazaki[1], Tomoki Makino[1], Yukinori Kurokawa[1], Makoto Yamasaki[1], Kiyokazu Nakajima[1], Shuji Takiguchi[1], Masaki Mori[1], Yuichiro Doki[1], Testuji Naka[1]

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INTRODUCTION: Constitutive activation of JAK/STAT pathway has been associated with tumor progression in various tumor. Recently, some reports showed that the STAT3 activation was induced by irradiation (IR) and can result in IR-resistance in various cancer. We previously reported that the overexpression of suppressor of cytokine signaling-1 (SOCS-1), which was cloned as a negative regulator of various cytokine signaling, using adenovirus vector (AdSOCS-1) has been a promising therapeutic approach for various cancer. Also in esophageal squamous cell carcinoma (ESCC), the overexpression of SOCS-1 showed a potent anti-tumor effect through targeting of JAK/STAT and FAK/ERK signaling pathway. From these results, we considered that SOCS-1 might have a possibility to overcome the IR-resistance for ESCC. The aim of this study is to evaluate the association between the activation of STAT3 and IR-resistance in ESCC and examine the antitumor effect of AdSOCS-1 & IR combined therapy. **METHODS:** First, we evaluated whether the STAT3 activation was induced by IR in ESCC cell lines and human ESCC sample. Second, we evaluated IR-resistance due to the STAT3 activation in ESCC cell lines by using pEB-Multi-constitutive-STAT3(c-STAT3) vector. We examined the colony forming assay between parent (TE8, TE9, TE14) and stably expression of c-STAT3 cells. Final, we evaluated cell growth inhibition effect of AdSOCS-1 & IR combined therapy *in vitro* and *in vivo*. As *in vivo* model, we examined the combined effect of AdSOCS-1 and IR (2Gy) by using TE14 xenograft mice (ICR nu/nu mice). **RESULTS:** First, the STAT3 activation was induced in ESCC cell lines according to IR(2Gy×3fraction), the expression of anti-apoptosis protein, such as Mcl-1 and Survivin which is a downstream of STAT3 also was enhanced. In human ESCC sample, the patient with preoperative radiation therapy significantly had high expression of phospho-STAT3(p-STAT3) compared to those without preoperative therapy in imunohistochemical analysis. Second, ESCC cells with stably expression of c-STAT3 showed a significant increase in colony forming ability after IR as compared with parent and mock cell. Therefore, the STAT3 activation might be associated with IR-resistance in ESCC cells. Final, we confirmed that AdSOCS-1 & IR combined therapy had better proliferation inhibitory effect and decreasing of colony forming ability. We also showed that AdSOCS-1 inhibited not only p-STAT3 but also the expression of anti-apoptosis protein (mcl-1, survivin) which was enhanced by IR, and induced apoptosis synergistically. As *in vivo* model, the combined therapy showed a significant anti-tumor effect compared to the IR alone and AdSOCS-1 alone group, and tumor growth was not observed. In the IR alone group, a marked development in the tumor after the treatment was observed, however, such a tendency was not observed in the combination treatment group **CONCLUSIONS:** The STAT3 activation was induced by IR and might be involved in IR-resistance in ESCC. Also, AdSOCS-1 & IR combined therapy may have a potent anti-tumor effect.

138. A Quantitative Imaging Toolbox to Evaluate the Effect of Endodomains on CAR Immune Synapse

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Introduction: The cytolytic immunological synapse (IS) in effector cells (T / NK / NKT) is a discrete structural entity formed after ligation of specific activation receptors to their targets, leading to target cell destruction by release of lytic granules. It is believed that killer cells have developed this tightly regulated process of IS formation to maximize delivery of an efficient cytolytic hit and reduce non-specific killing. Disruption of any one of the multi-step components of IS formation can lead to dire consequences evidenced in autoimmune diseases, or in primary immunodeficiencies. The dynamics of these highly preserved synapse components in formation of an "engineered" chimeric antigen receptor (CAR) IS have not been established and the precise downstream effect of varying costimulatory domains on these critical cytotoxic parameters in CAR expressing cells is therefore unclear. Our work focuses on understanding the stepwise IS formation in CAR T-cells using highly quantitative high and super resolution imaging techniques. High resolution imaging reveals critical differences between CARs harboring CD28 or 4-1BB costimulatory endodomains and their way of target recognition and killing based on the re-arrangement of cell structural components like intercellular adhesion molecule-1 (ICAM-1) and the subsequent recruitment of CD95/Fas or perforin-mediated cytotoxic pathway. **Methods**: High and super resolution fixed and live cell microscopy platforms are used to image CAR T cell-target conjugates in the x, y, z and t dimensions. Imaging parameters include direct evaluation of CAR engagement at the IS and the timing and arrangement of cytolytic T cell synapse components like ICAM-1 and LFA-1, F-actin, microtubule organizing center (MTOC), lytic granules and Fas. **Results**: Using a quantitative imaging toolbox to interrogate the CAR IS we found that 4-1BB. zeta CAR T-cells increased surface levels of CD95/Fas and recruited FasL from intracellular compartments to the cell surface resulting in enhanced Fas-mediated apoptosis in CAR T-cells. Furthermore, activation of the NKκB pathway by 4-1BB signaling led to upregulation of surface ICAM-1, resulting in formation of stable IS with target cells with increased F-actin polymerization (Figure 1) and MTOC polarization. The expression of surface ICAM-1 was normal in CD28. zeta CAR T-cells but remained high in "third generation" (CD28.4-1BB.zeta CAR). So, ICAM-1 upregulation by CAR-derived 4-1BB signaling may promote a more "sticky" immune synapse with target

cells expressing the ICAM-1 ligand LFA-1 providing the possibility for manipulation of this pathway for a more effective CAR in the right context.Conclusions: Using a quantitative imaging toolbox we are able to decipher at a single cell level, important differences in cytotoxic pathways mediated by CD28.zeta or 4-1BB.zeta CARs that can have direct implications on CAR mediated cytotoxicity.

Figure 1

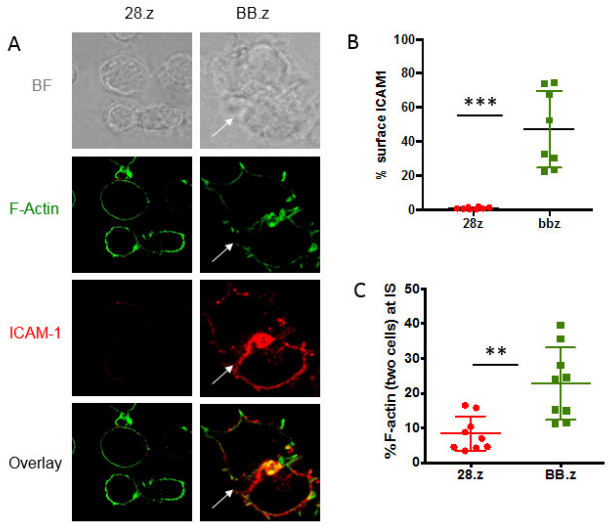

Figure 1. 4-1BB.z CAR T cells show enhanced expression of surface ICAM-1 and F-actin at intracellular contact points compared to 28.z CAR. Representative images of emerald GFP (green) tagged CD5 CAR T cells with 28.z (left panel) or 4-1BB.z (right panel) are stained with antibodies specific for F-actin (Phalloidin, green) and ICAM-1 (red) and imaged as z stacks using a Leica SP8 laser scanning confocal microscope. n=3 experiments. $^{**}p \leq 0.01$.

Figure 1. 4-1BB.z CAR T cells show enhanced expression of surface ICAM-1 and F-actin at intracellular contact points compared to 28.z CAR. Representative images of emerald GFP (green) tagged CD5 CAR T cells with 28.z (left panel) or 4-1BB.z (right panel) are stained with antibodies specific for F-actin (Phalloidin, green) and ICAM-1 (red) and imaged as z stacks using a Leica SP8 laser scanning confocal microscope. n=3 experiments.

139. Intranasal Delivery of a Novel Anticancer Compound, Obtusaquinone, for the Treatment of Pediatric Gliomas

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<Introduction: Pediatric high-grade gliomas (HGGs), including glioblastoma and diffuse intrinsic pontine glioma (DIPG), have a remarkably different molecular and genetic profile compared to their adult counterparts; therefore, existing data on the treatment and management of adult HGGs cannot be translated to pediatric tumors. Few studies tried to unravel the biological and molecular properties of pediatric HGGs, however, due to the insufficient body of data, the standard of care for pediatric HGGs is still based on adult studies, hence is suboptimal. New therapeutics and management plans are highly needed for this population of patients.><Through high-throughput screening using naturally secreted Gaussia luciferase (Gluc) as a viability marker, delivered through a lentivirus vector, we have discovered the anti-cancer properties of the natural compound obtusaquinone (OBT). Here, we aimed to evaluate the effect of OBT on patient-derived pediatric gliomas. We hypothesized that by delivering the compound intra-nasally, we could bypass the limiting properties of the blood-brain barrier and therefore achieve an optimal anti-tumor effect, lower the therapeutic doses of the drug, and eliminate possible side effects, as is the case for systemic delivery.><Methods: We first investigated the cytotoxic effect of OBT on different pediatric GBM and DIPG cells in culture by implementing different cell viability and colony formation assays. Cells were engineered by a lentivirus vector to stably express an apoptosis reporter which we have developed based on Gluc. In this case, Gluc is caged inactive inside the cells. Upon caspase activation, Gluc is cleaved, activated and secreted, which can then be measured in the conditioned medium of cells. We also evaluated the mechanism of action by immunoblotting for pathways related to reactive oxygen species (ROS) induction and apoptotic cell death. Finally, we engineered pediatric GBM and DIPG cells to stably express firefly luciferase (Fluc) and implanted them intracranially into the brains of athymic nude mice using typical coordinates for pediatric GBM or DIPG. Once tumors were formed, mice were divided into 2 groups, which received an intranasal injection of either DMSO vehicle or OBT (750 µg/kg/day, a 10-fold lower dose compared to our previous study with systemic injection). Tumor growth and response to OBT therapy was monitored by in vivo Fluc bioluminescence imaging and was confirmed by ex vivo histological analysis.><Results: Obtusaquinone induced cell death in a dose-dependent manner in different GBM and DIPG cell lines and patient-derived cells. OBT induced ROS generation, leading to pediatric glioma cell death. In vivo, intranasal administration of a relatively low dose of OBT reduced tumor growth, leading to a significant increase in survival rate ($P < 0.05$).><Conclusion: We show that OBT is a potent agent for the treatment of pediatric GBM and DIPG. We also demonstrate that intranasal delivery of OBT is a minimally invasive procedure, which could bypass the blood-brain barrier, requiring 10-fold lower dose, and yielding an improved tumor response. Intranasal delivery could be implemented to other drugs for the treatment of brain tumors, including pediatric gliomas since it diminishes the systemic toxicity or hypersensitivity reactions.

140. Poly(Beta-Amino Ester)-Mediated Cancer-Specificity and Transarterial Delivery to Improve Gene Therapy Targeting to Hepatocellular Carcinoma Lesions

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of Material Science and Engineering, Johns Hopkins University, Baltimore, MD, [8]Depts. of Chemical and Biomeolecular Engineering, Johns Hopkins University, Baltimore, MD, [9]Depts. of Neurosurgery, Oncology, and Ophthalmology, Johns Hopkins University School of Medicine, Baltimore, MD

Introduction: Hepatocellular carcinoma (HCC) is one of the most lethal cancers in the US and worldwide. Less than 30% of HCC patients are eligible for liver transplantation, but little progress has been made in the past decades towards developing another curative treatment option. Nevertheless, the differential vascularization between normal parenchyma (portal vein) and HCC (hepatic artery) enables local delivery of chemotherapy through the hepatic artery. Despite the regio-selectivity provided by the transarterial route, cytotoxic agents still cause critical damage to healthy liver cells. These treatments have also proven ineffective, preventing transarterial drug administration from being incorporated in the curative care of HCC patients. Poly(Beta-amino ester) (PBAE) nanoparticles (NPs) have demonstrated biomaterial-mediated specificity in DNA delivery to cancer cells. Here we propose to evaluate the synergistic effect of regio-selective delivery and PBAE-mediated specificity to improve HCC targeting. **Methods:** A human hepatocyte line and nine human HCC lines were separately transfected with varied PBAE:GFP DNA polyplex NP formulations. Cell viability was assessed by MTS and transfection efficacy (eGFP positive) by flow cytometry. Co-culture of HCC cells (RFP positve) and hepatocytes (RFP negative) were also transfected with eGFP, and cancer-specificity was assessed by evaluating GFP/RFP co-expression using flow cytometry. To evaluate efficacy *in vivo*, an orthotopic human HCC xenograft model was established in athymic rats. Human HCC cells were implanted under the liver capsule through an open laparotomy. Transarterial injections of PBAE-NPs are performed by injection into the proper hepatic artery. **Results:** High transfection efficacy to all the liver cancer lines, but not to the hepatocytes was observed with an optimized PBAE-NP formulation. Each HCC line had a higher eGFP expression (eGFP positive %) than the hepatocyte line ($P < 0.01$). In addition, this same formulation preserved the viability of hepatocytes, which remained above 80%. In the HCC and hepatocyte co-culture model, NP-mediated cancer selectivity could also be observed (95.4% ± 0.5 eGFP expression in the HCC line vs 35.6% ± 0.3 in the hepatocyte line; $P < 0.01$). An orthotopic human HCC xenograft model was successfully established in athymic rats and HCC lesions could be observed after 5 weeks of the tumor implantation procedure. **Conclusion:** Select PBAE-NP formulations enabled preferential DNA delivery to varied HCC cell lines, sparing healthy hepatocytes and preserving their viability. Additionally, an orthotopic human HCC xenograft model was successfully established in athymic rats, which allows for evaluation of gene therapy delivery with PBAE-NPs via hepatic artery.

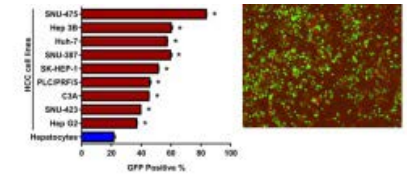

Fig. 1 Effective and cancer-specific DNA delivery to HCC cell lines over healthy hepatocytes *in vitro*.

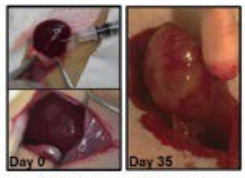

Fig. 2 Established orthotopic human HCC xenografts in athymic rats

141. A Murine Model of Suicide Gene Therapy Against Brainstem Tumors Shows Efficacy in the Absence of Inflammatory Toxicity, Suggesting Use of Suicide Gene Therapy Vectors as Treatments for Diffuse Intrinsic Pontine Glioma (DIPG)

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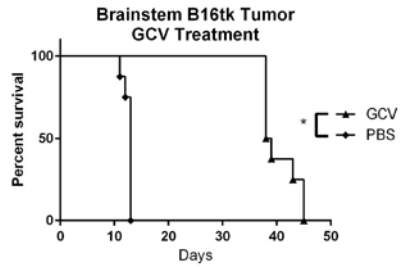
Diffuse intrinsic pontine glioma (DIPG), a rare pediatric brainstem tumor, is the leading cause of death among pediatric brain malignancies. Unfortunately, over the past several decades no significant progress has been made, leading our lab to explore the use of suicide gene therapy. The brainstem has uniquely sensitive anatomy, creating the possibility of toxic inflammation. Additionally, clinically-used agents for DIPG include conventional radiation therapy (RT) and dexamethasone (DEX), each of which can alter viral and immune-mediated therapies. In this study we found that a variety of human and murine glioma cell lines are infectable *in vitro* with a replication-competent Moloney murine leukemia virus (MoMLV) (currently in Phase 2/3 clinical trials) and susceptible to ganciclovir (GCV)-mediated killing via the HSV thymidine kinase (tk) suicide gene. A B16 melanoma cell line expressing HSVtk was implanted into the brainstem of mice, where GCV therapy extended median survival from 13 days to 38.5 days (p=.0006) with no signs of toxic inflammation. Pre-treatment with RT and DEX did not alter therapeutic efficacy. We have previously shown that GCV/HSVtk therapy is mediated by NK and CD8 cells in subcutaneous tumors, and we are investigating if a similar mechanism is involved in this brainstem model. These preclinical data pave the way for novel clinical trials to treat DIPG.



142. Evaluation of the Glypican-3 Promoter for Transcriptional Targeting of Hepatocellular Carcinoma

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Background: Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver and represents the third most common cause of death from cancer globally. Limited therapeutic options, especially in an advanced stage, combined with the presence of underlying liver dysfunction in most of the patients limit its treatment. Targeted gene therapy may be a promising treatment in this setting. Transcriptional targeting of cancer can be achieved using promoters preferentially active in tumor cells (tumor specific promoters (TSPs)). Glypican 3 (GPC3) is an oncofetal protein belonging to the proteoglycan family and is highly expressed in HCC and not in normal or cirrhotic liver. Given the HCC-specific nature of GPC3 expression, we hypothesized that the promoter for this gene should be preferentially active in HCC. Methods: To test this hypothesis we evaluated the potential of the glypican 3 promoter for transcriptional targeting of HCC. The promoter of alpha fetoprotein (AFP), an established HCC-specific promoter, was used for comparison. The activity of these promoters in a panel of normal liver, HCC and non-HCC cell lines was evaluated by correlating it with the expression levels of corresponding genes using real time rtPCR. The GPC3 promoter was isolated from HCC cells and cloned into an expression vector to drive the expression of enhanced yellow fluorescence protein (eYFP). The expression level of eYFP for each cell type was quantified using the flow cytometer and normalized against the ubiquitous cytomegalovirus (CMV) promoter. Results: GPC3 was detected in 5 out of 7 HCC cell lines studied. This was in line with human patient tumours where GPC3 expression has been reported in up to 50-80% of HCC. In contrast, the expression of GPC3 was not detected in normal liver and most other non-HCC tumor cell lines. Interestingly a number of non-HCC cell lines including HeLa, LNCaP (prostate), 92.1 (melanoma) and Caco-2 (colon) also showed significant levels of GPC3 expression. Next, we assessed whether the GPC3 promoter could limit transgene expression to HCC which express GPC3. We found that the promoter was able to preferentially induce the expression of eYFP in HCC cells when compared to normal liver cells; this preference was comparable and in some cases better than the selectivity of the AFP promoter. While the GPC3 promoter appeared to allow selective expression in HCC compared to normal liver cells, we did detect GPC3 driven transgene expression in some non-HCC cancer cells, which was also seen with the AFP promoter in our hands. Conclusion: We have demonstrated that the promoter for GPC3 is active in the majority of HCC and not in normal liver and can be used to selectively target HCC cells with gene therapy; however as with other promoter driven systems, additional targeting strategies may be required if treatment is delivered systemically.

143. Targeting XPC/ERK/Snail/E-Cadherin Pathway, miR-346 Facilitates Cell Growth and Metastasis, and Suppresses Cell Apoptosis in Human Non-Small Cell Lung Cancer

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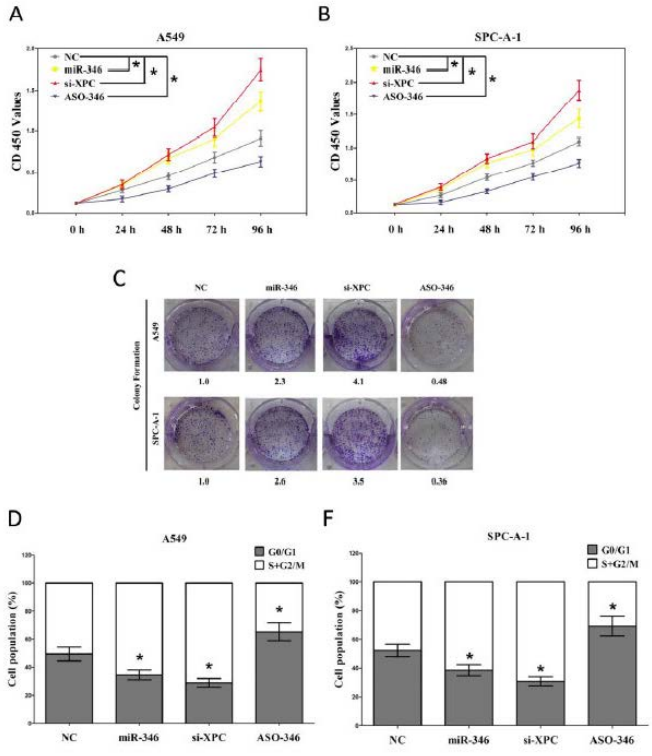
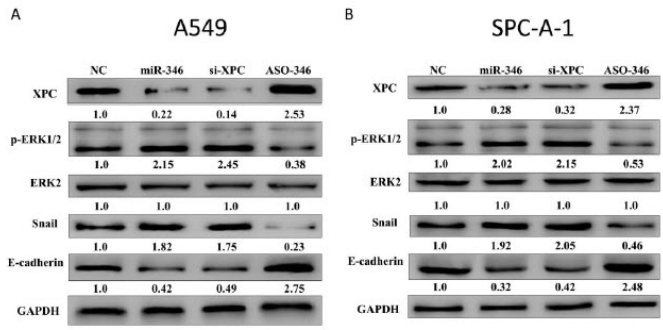
Determinants of growth and metastasis in cancer remain of great interest to define. MicroRNAs (miRNAs) havefrequently emerged as tumor metastatic regulator by acting on multiple signaling pathways. Here we report thedefinition of miR-346 as a novel oncogenic microRNA that facilitates non-small cell lung cancer (NSCLC) cellgrowth and metastasis. XPC, an important DNA damage recognition factor in nucleotide excision repair wasdefined as a target for down-regulation by miR-346, functioning through direct interaction with the 3'-UTR ofXPC mRNA. Blocking miR-346 by an antagomiR was sufficient to inhibit NSCLC cell growth and metastasis, aneffect that could be phenol-copied by RNAi-mediated silencing of XPC. In vivo studies established that miR-346overexpression was sufficient to promote tumor growth by A549 cells in xenografts mice, relative to controlcells. Overall, our results defined miR-346 as an oncogenic miRNA in NSCLC, the levels of which contributed totumor growth and invasive aggressiveness.





144. Long Intergenic Non-Coding RNA 00511 Facilitates Non-Small Cell Lung Cancer Progression by Binding to EZH2 and Suppressing p57

Cheng-Cao Sun, Shu-Jun Li, De-Jia Li

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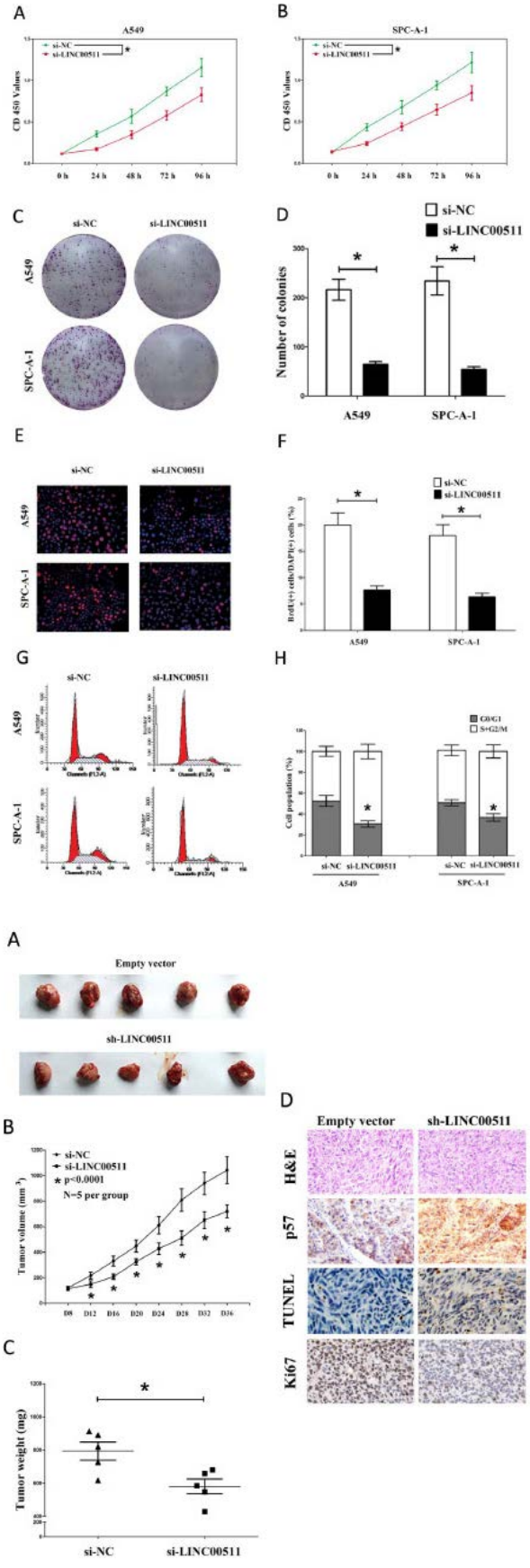
Long non-coding RNAs (lncRNAs) play crucial roles in arcinogenesis. However, the function and mechanism of lncRNAs in human non-small cell lung cancer (NSCLC) are still remaining largely unknown. Long intergenic non-coding RNA 00511 (LINC00511) has been found to be up-regulated and acts as an oncogene in breast cancer, but little is known about its expression pattern, biological function and underlying mechanism in NSCLC. Herein, we identified LINC00511 as an oncogenic lncRNA by driving tumorigenesis in NSCLC. We found LINC00511 was up-regulated and associated with oncogenesis, tumor size, metastasis and poor prognosis in NSCLC. Moreover, LINC00511 affected cell proliferation, invasiveness, metastasis and apoptosis in multiple NSCLC cell lines. Mechanistically, LINC00511 bound histone methyltransferase enhancer of zeste homolog 2 [(EZH2, the catalytic subunit of the polycomb repressive complex 2 (PRC2), a highly conserved protein complex that regulates gene expression by methylating lysine 27 on histone H3], and acted as a modular scaffold of EZH2/PRC2 complexes, coordinated their localization, and specified the histone modification pattern on the target genes, including p57, and consequently altered NSCLC cell biology. Thus, LINC00511 is mechanistically, functionally, and clinically oncogenic in NSCLC. Targeting LINC00511 and its pathway may be meaningful for treating patients with NSCLC.



Cell Therapies I (Pluripotent, Hematopoietic, Endothelial, Beta and Neural Cells)

145. Preliminary Tolerability of iPSC-Derived RPE on PLGA Scaffold Following Subretinal Implantation in RNU Nude Rats

Ewa Budzynski[1], Brittany Mancl[1], Brian Christian[1], Michael T. Nork[2,3], Paul E. Miller[2,3], Steve Sorden[1], Arvydas Maminishkis[4], Vladimir Khristov[4], Jha Balendu[4], Kapil Bharti[4]

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Dysfunction of retinal pigment epithelial cells (RPE) is an important factor in development of age related macular degeneration (AMD). RPE support photoreceptor function, and degeneration of RPE leads to retinal degeneration and vision loss. RPE replacement therapy has the potential to stop such degeneration. The purpose of this study was to evaluate the ocular tolerability of AMD patient-specific induced pluripotent stem cell (iPSC)-derived RPE on poly lactic-co-glycolic acid (PLGA) scaffold following subretinal implantation in rats. Male Crl:NIH-Foxn^{1rnu} (RNU nude) rats underwent sham surgery in the right eyes or were administered implant of AMD iPSC-derived RPE on PLGA scaffold that was 0.5 mm in diameter via subretinal surgery. The rats were observed for 6 weeks. Body weight, food consumption, clinical observations, and slit lamp bio-microscopy, indirect ophthalmoscopy, and histological evaluation of the eyes were performed.

AMD iPSC-derived RPE as monolayer on a scaffold were generally well tolerated as assessed by ophthalmic examination and had no effect on general health. Histologically, 7/15 eyes given iPSC-derived RPE had foci of minimal hyperplasia of the RPE 6 weeks after implantation. PMEL17- and STEM121-immunopositive cells were present in 6/15 eyes given iPSC-derived RPE . PMEL17- and STEM121-immunopositive cells were colocalized and generally occurred within the foci of RPE hyperplasia or the adjacent RPE in the region of the subretinal surgical site. No PMEL17- or STEM121immunopositive cells were identified in sham-treated animals or in animals sacrificed at an unscheduled interval. The greater eye manipulation required to insert the scaffold, compared with the sham procedure was associated with more corneal abnormalities. In conclusion, subretinal implantation of AMD patient specific iPSC-derived RPE on PLGA scaffold was well tolerated by the RNU nude rat. Six weeks after implantation, the cells appeared to be incorporating into the native RPE.

146. A Novel Construction of Lentiviral Vectors for Eliminating Tumorigenic Pluripotent Stem Cells

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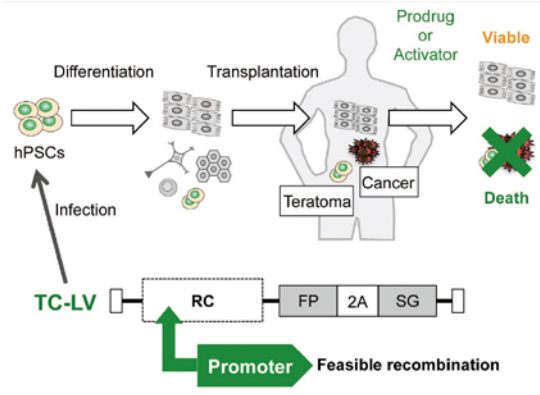
The risk of tumor formation poses a challenge for human pluripotent stem cell (hPSC)-based transplantation therapy. Because conventional strategies, e.g., establishing safe hPSC lines, improving the efficiency of differentiation induction, and indirectly ensuring the safety of products, are unlikely to completely overcome this issue due to the intrinsic characteristics of hPSCs, innovative safety approaches should be developed. In this regard, we previously developed "adenoviral conditional targeting" that securely isolated target cells (Mol Ther. 14: 673-683. 2006), and a novel "oncolytic virus" strategy that specifically eliminated undifferentiated cells, thereby inhibiting teratoma formation after hPSC transplantation (Mol Ther Methods Clin Dev. 2, 15026, 2015). In addition, a novel comprehensive approach would be to engineer a suicide gene into hPSCs. However, specific and total elimination of tumorigenic hPSCs by suicide genes has not been achieved because no methodology currently exists for testing multiple candidate transgene constructs. We present a novel method for efficient generation of tumorigenic cell-targeting lentiviral vectors (TC-LVs) with diverse promoters upstream of a fluorescent protein and suicide genes. Our two-plasmid system achieved rapid and simultaneous construction of five TC-LVs with five different promoters. Ganciclovir exerted remarkable cytotoxicity in herpes simplex virus thymidine kinase (HSV-tk)-transduced hPSCs, and perfect specificity for undifferentiated cells was achieved using the survivin promoter (TC-LV.Surv). Moreover, ganciclovir treatment completely abolished teratoma formation by TC-LV.Surv-infected hPSCs transplanted into mice, without harmful effects. Thus, TC-LV can efficiently identify the best promoter and suicide gene for specific and complete elimination of tumorigenic hPSCs, facilitating the development of safe regenerative medicine. In this 2017 ASGCT meeting, we present in vivo results as well as the detailed method of TC-LV construction.



147. A Novel Feeder- and Serum-Free Culture System to Derive Human Retinal Pigment Epithelium from Pluripotent Stem Cells

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The neural retina activity relies on retinal pigment epithelium (RPE) functions and its deficiency give rise to several diseases, of which most of them result in visual impairments or blindness. The ability to generate hRPE for disease modelling, drug screening or transplantation is particularly worth to answer these important challenges. Here we present an easy, reliable, and serum-free method to reliably generate hRPE from induced pluripotent stem cells (iPSCs) in culture. Starting from feeder-free culture conditions we established a simple three-step protocol able to induce pigmented foci as early as 18 days after differentiation. After a first step consisting on the formation of embryonic body-like aggregates in floating condition, the neuroephitelium induction follows upon plating, then the third phase commit the neural cells to RPE fate. Extensive characterization was performed to confirm expression of typical RPE markers by polymerase chain reaction, immunohistochemistry and enzyme-linked immuno-sorbent assay. Ultrastructure was verified by electron microscopy and phagocytosis assay assessed phagocytic capacity of RPE cells by Western blotting against Rhodopsin after photoreceptors outer segments (POS) seeding. iPS-RPE cells exhibited pigmentation and cobblestone-like morphology and expressed mRNAs of typical RPE markers such as RPE65 (visual cycle), MERTK (phagocytosis), BEST1 (ion homeostasis), ZO-1 (tight junctions) or OTX2 (transcription factor). Protein presence of some of these markers was assessed by immunohistochemistry and phagocytosis assay showed slow kinetics of POS internalization. Electron microscopy revealed polarized iPS-RPE cells and PEDF and VEGF secretion level ranged in commonly reported values (407.5±20.98 and 19.70±1.934 ng/24hr/cm^2 respectively, MEAN±SEM). Finally, RPE cells were infected with lentivirus bearing different promoters in eGFP construct as an insight into iPS-RPE cells response to LV-based gene therapy. The specificity of the promoter activity is under quantification. The presented protocol provides a quick and consistent method to generate robust hRPE from pluripotent stem cells. By combining a CRISPR approach to mutate hiPSCs line and our RPE differentiation protocol, we aim at producing *in vitro* model for RPE deficient-induced retinal diseases.

148. Genome-Wide Definition of Regulatory Regions and Transcripts During the Transition from Pluripotent to Neural-Restricted Stem Cells

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Human fetal-derived neural stem/progenitor cells (hfNSCs) are under clinical evaluation for several neurodegenerative diseases. These cells display a favorable safety profile but require immunosuppression upon allogeneic transplantation in patients. In this scenario, obtaining *bona-fide* neural stem cell (NSC) populations from human induced pluripotent stem cells (hiPSCs) may be relevant for the development of autologous *ex-vivo* gene therapy approaches to treat neurological disorders. We have recently generated a collection of hiPSC-derived NSCs (hiPS-NSCs) sharing molecular, phenotypic and functional identity with hfNSCs, which we used as a "gold standard" in a side-by-side comparison to validate the hiPS-NSC phenotype *in vitro* and *in vivo*. We gave proof-of-principle of the potential application of hiPS-NSCs in autologous *ex vivo* gene therapy protocols for metachromatic leukodystrophy (MLD), a fatal neurodegenerative LSD caused by genetic defects of the arylsulfatase A (ARSA) enzyme. Patient-specific hiPS-NSCs, genetically modified to express supraphysiological ARSA levels, were intracerebrally transplanted into neonatal and adult immunodeficient MLD mice restoring long-lasting enzymatic supply in the whole CNS and significantly reducing the pathological sulfatide storage. The transcriptional and epigenetic mechanisms underlying hiPSC commitment towards the neural lineage need to be investigated to optimize the production and define the safety profile of hiPS-NSCs in the perspective of their potential clinical application. We are currently performing a genome-wide mapping of regulatory elements, integrating RNA-seq and ChIP-seq data to provide: (i) the comprehensive profile of genes differentially expressed among hiPSCs, hiPS-NSCs and hfNSCs; (ii) the broad description of promoter and enhancer usage characterizing the transition from pluripotent to neural restricted stem cells; (iii) the comparison of regulatory elements activated in hiPS-NSCs and hfNSCs. The identification of markers of critical steps in neural lineage commitment will aid strategies for increasing efficiency and consistency of hiPSC to neural differentiation. Furthermore, the definition of hiPS-NSC epigenetic and transcriptional signatures in comparison to hfNSCs is an essential step to better define their cell identity and safety profile.

149. Exploring the Potential of Anti-Mouse c-kit Chimeric Antigen Receptor (CAR)-T Cells as Conditioning for Bone Marrow Transplantation

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INTRODUCTION: Anti-c-kit and anti-CD45 antibodies including saponin conjugates achieve efficient conditioning by targeting bone marrow (BM) hematopoietic stem cells (HSC). We explored the potential of anti-c-kit chimeric antigen receptor (CAR)-T cells as a BM preparative regimen. There are pros and cons to cell-based HSC conditioning. The pros are that CAR-T cells may be modified to achieve more precise tissue trafficking and cell targeting. CAR-T cells can also be equipped with suicide genes allowing them to be deleted following conditioning or if toxicities are encountered. The cons are cell therapies are more complex to manufacture and may prove difficult to eliminate. **METHODS**: Mouse spleen or human peripheral blood T cells pre-activated with anti-CD3/28 antibodies were transduced with lenti- or retroviral vectors expressing variable regions of anti-mouse or anti-human c-kit immunoglobulin, respectively, and intracellular signaling domains of second generation CAR cassettes including CD28 and CD3ζ (>90% of T cells expressed CAR). *In vitro* co-culture assays were performed by mixing CAR-T with mouse or human HSC in nitrocellulose medium for 10 days. For some *in vivo* studies, co-transduction of murine CXCR4 overexpression was used. **RESULTS**: Co-culture of mouse BM with CAR-T cells *in vitro* specifically triggered CAR-T cell generation of IFNγ (101.0 vs. 0.3 ng/mL; $p < 0.01$) accompanied by specific depletion of the c-kit positive population of BM cells (1.6% from 10.5%; $p < 0.01$) and suppressed BM colony formation (0 vs. 55 colonies per dish; $p < 0.001$). Similar *in vitro* results were with human CAR-T cells and mobilized human $CD34^+$ HSC (enhanced secretion of IFNγ of 10.5 vs. 2.1 ng/mL; $p = 0.01$; and decreased colony formation of 45 vs. 84 colonies per dish; $p < 0.01$). Initial studies of mouse CAR-T cells injected intravenously into C57B6 mice showed that no CAR-T cells were detected in BM, nor was there any change in the $c\text{-}kit^+$ population in the BM (6.9% compared to 7.8% in untreated mice; $p = 0.70$). We enhanced trafficking to BM by co-transducing murine CXCR4 together with the anti-c-kit CAR. CAR-T cells over-expressing CXCR4 detectably trafficked into BM (1.8% of total BM cells compared to 0.0% without CXCR4 overexpression) resulting in significant reduction of $c\text{-}kit^+$ population to 1.7% from baseline 6.8% ($p = 0.02$). Curiously, there was recovery of the $c\text{-}kit^+$ population over 7 days despite persistence of CAR-T cells in BM. In preliminary experiments, not yet complete, where syngeneic donor BM cells were transplanted (CD45.1 into CD45.2) a few days after autologous CAR-T injection (without any other conditionings), there was significant engraftment of donor cells at one month compared to mice similarly transplanted without CAR-T cell treatment (>2.0% vs. 0.2%). **CONCLUSION**: Mouse or human anti-c-kit CAR-T cells can target mouse or human HSC *in vitro*. Mouse anti-c-kit CAR-T cells fail to traffic into the BM *in vivo* unless overexpressing CXCR4. While these results are encouraging as the potential candidate of BM conditioning in non-malignant hematological or immunedeficient diseases, much additional work is necessary, as anti-c-kit CAR-T cells are much less effective than anti-c-kit antibodies for BM conditioning. Nonetheless, one important outcome of our study more generally applicable to CAR-T cell technology is that failure of T cell trafficking to a target tissue including tumors may be a problem that is amenable to maneuvers that increase trafficking through forced expression of receptors mediating chemotaxis to the target tissue.

150. Development of a Stem Cell Gene Therapy for Sanfilippo Syndrome B

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Sanfilippo syndrome type B (Mucopolysaccharidosis type IIIB; MPS IIIB) is a lysosomal storage disorder (LSD) affecting primarily the brain and is characterized by profound intellectual disability, dementia, and a lifespan of about twenty years. The cause is a mutation in the gene encoding α-N-acetylglucosaminidase (NAGLU), a lysosomal enzyme, leading to the deficiency of NAGLU and accumulation of heparan sulfate (HS). Obstacles to treatment for MPS IIIB include regular intracranial injection of enzyme and the blood brain barrier (BBB). Sadly, even bone marrow transplantation is ineffective in treating MPS IIIB. We address these issues using a stem cell gene therapy approach in a Naglu-/- mouse model. Naglu-/- mouse embryonic fibroblasts were reprogrammed to induced pluripotent stem cells (iPSCs), then differentiated to neural stem cells (iNSCs). Using lentiviral transduction, Naglu was overexpressed in Naglu-/- iNSCs. Here we report that Naglu overexpressed in iNSCs is capable of being taken up by deficient cells. Not only can enzyme be found inside of Naglu deficient cells, but Naglu can reduce lysosomal volume compared to deficient cells in vitro. iNSCs overexpressing Naglu were injected into the brains of 1 day old Naglu-/- mice. Each Naglu-/- mouse injected showed evidence of engraftment. Several sections possessed Naglu activity greater than or equal to heterozygous controls, marked decrease in GFAP and Lamp-1, and Naglu activity was detected distal to injection sites. The results suggest that engineered iNSCs could be used as a vehicle to treat MPS IIIB.

151. Developing Cell and Gene Therapy to Treat Friedreich Ataxia

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Friedreich ataxia (FRDA) is a debilitating neurodegenerative disorder, affecting 1 in 29 000 individuals of Caucasian descent. Disease onset typically at around 10-15 years of age and is primarily characterised by progressive gait and limb ataxia. This results in individuals with FRDA to experience an ongoing loss of motor coordination and becoming wheelchair- dependent within 15 years after disease onset - decreasing their quality of life. Individuals with FRDA also suffer from other disease symptoms such as hypertrophic cardiomyopathy, with complications with the heart being the most common cause of death in FRDA with life expectancy decrease to an average of 37 years old. As there is no current treatment which can cure or slow the neurodegeneration inherent to FRDA, individuals with FRDA manage disease symptoms by undergoing a myriad of symptomatic treatment. The development of new and more effective treatments capable of slowing or halting disease progression is therefore essential to improve the quality of life of the individuals living with FRDA. In most cases, FRDA is caused by a homozygous GAA trinucleotide repeat expansion within intron 1 of *FXN* which encodes for frataxin, a nuclear-encoded mitochondrial protein. As the expansion only reduces the level and does not alter the frataxin protein sequence and structure, an increase in frataxin expression is predicted to be therapeutically beneficial to individuals with FRDA. To investigate the therapeutic potential of bone marrow transplantation (BMT) as a cell-based therapy for FRDA, the transplantation of either wild type (corrected mice) or FRDA (non-corrected mice) BM stem cells into the YG8 mice resulted in successful reconstitution of the haematopoietic system with donor BM-derived stem cells at eight weeks post-BMT. Neuro-behavioural assays however showed no significant difference in the motor coordination, locomotor activity and limb flexion response between corrected and non-corrected mice at all time points tested. In order to identify if the transplantation of BM stem cells itself was beneficial, YG8 mice were then tested on the various neuro-behavioural assays and showed a significant decrease in motor coordination, locomotor activity and limb flexion response as the mice aged. These observations suggested that transplantation of either the wild type or FRDA BM prevented the decline in motor functions and that both types of BM stem cells are beneficial for motor behavioural outcomes in FRDA disease progression. GFP-positive cells were also identified in the dorsal root ganglia (DRGs) and spinal cord tissues, indicating successful engraftment of donor BM-derived stem cells in major sites of neuropathology post-BMT and demonstrating low-level chimerism. Western blotting analysis showed a significant increase in the mature frataxin protein in spinal cord tissues at 3 months post-BMT. Immunofluorescence studies also showed increased neuronal marking in the DRGs of corrected mice compared to non-corrected mice, in particular the proprioceptive neurons which are highly affected in individuals with FRDA. These results together illustrate the corrective potential of BMT to treat FRDA and provide an avenue for delivering therapeutic viral vectors for autologous gene therapy.

152. Selection and Purification of CD34+ Cells from Cryopreserved Cord Blood Units Using CliniMACS Plus and CliniMACS Prodigy

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Umbilical cord blood is a critical source of hematopoietic progenitor and stem cells (HPSC) that could support the reconstitution of the hematopoietic system post transplantation. The relatively low number of CD34+ cells in a cord blood unit (CBU) remains a major obstacle for the engraftment and rapid immune reconstitution in adult patients. Selection and enrichment of cord blood derived CD34+ cells will permit the ex vivo expansion and/or engineering of this pluripotent cellular subset to support immune reconstitution and the potential curative treatment of various diseases. Encouraging results for purification of CD34+ cells from freshly mobilized peripheral blood and fresh cord blood have been reported. However, purification of CD34+ cells from cryopreserved CBU remains challenging. We evaluated the purification of CD34+ cells from cryopreserved CBUs using both the semi-automatic CliniMACS Plus and the new highly automated CliniMACS Prodigy instruments. For the purification of cryopreserved CBU on CliniMACS Plus, red blood cells and platelets were removed by using either the size-exclusion based LOVO cell washer or centrifugation, followed by the incubation with CD34+ antibody-coated magnetic beads. The purification was subsequently performed on CliniMACS Plus using the standard "normal scale" selection process. On the Prodigy, all the cell washing, bead incubation and purification steps were accomplished using the automated "Cord Blood CD34" program. The recovery of CD34+ cells from Prodigy (n=4) was significantly higher than that from CliniMACS Plus (n=3) (43 to 51.5% vs 19.5 to 25%, p=0.0001), with comparable CD34+ cell purity (60.3 to 88.6% vs 91.0 to 91.6%, p=0.06). CBUs yielding the lowest CD34+ enrichment from the Prodigy runs correlated with the lowest initial CD34+ cell content in the cryopreserved cord blood units. In addition to higher CD34+ cell recovery, Prodigy requires markedly less hands-on operator time and experience. We will investigate the subsequent ex vivo expansion potential of the CD34+ cells selected with Prodigy and validate whether it is a suitable platform for this application.

154. Improved *In Vitro* Models of the Human Blood-Brain Barrier (BBB) Using Endothelial Cells Derived from Induced Pluripotent Stem (iPS) Cells for Testing Therapeutics

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The blood-brain barrier (BBB) is primarily composed of highly specialised brain microvascular endothelial cells (BMECs) sharing the basal lamina with pericytes and end processes of astrocytes. The BBB tightly controls the exchange of molecules and cells between the brain and the blood. Although the BBB successfully maintains the brain microenvironment, it also blocks beneficial therapeutics for diseases of the central nervous system (CNS). The tight junction between the BMECs is the recognised phenotype of the BBB and is empirically defined by high trans-endothelial electrical resistance (TEER) and low permeability to paracellular markers. Efforts to model the BBB *in vitro* have been ongoing for many decades. The early *in vitro* BBB models were mainly constructed using primary BMECs from animal brain tissue, including rat, mouse, porcine and bovine cells. The next generation BBB models included co-culture of BMECs with different combinations of other cells of the BBB, such as pericytes and astrocytes, to improve the BBB properties. Models derived from animal tissue have proven extremely useful in studying various aspects of the BBB, such as developmental and regulatory mechanisms and assaying drug permeability. However, they generally have relatively low TEER values and high permeability to paracellular markers. We have produced improved *in vitro* models of the BBB using endothelial cells differentiated from human induced pluripotent stem (iPS) cells following a published protocol (Lippmann et *al*., Nat Biotechnol 30 783-791, 2012). Three different clones of wildtype (WT 4603, WT 19-9-7th and WT AD3-CL1) and a single clone of Spinal Muscular Atrophy type I (SMA I) iPS cells were differentiated into endothelial cells, characterised and assessed for proficiency to form BBB models. The integrity of the models was evaluated using TEER, expression of tight junction protein occludin, and permeability to paracellular markers lucifer yellow (LY) and sodium fluorescein (NaF). For comparison, the TEER of the most widely used *in vitro* model of the human BBB, made of the primary human brain endothelial cell line hCMEC/D3 (D3), was used.

The TEER values of WT 4603 and WT 19-9-7th 48 hours after seeding are comparable to the value reported for co-culture models using iPS endothelial cells, pericytes and astrocytes (Lippmann *et al*., Sci Rep 4 : 4160, 2014) and over 60-fold higher than in D3 cells (**A**). The permeability to LY and NaF was 40-fold and 3-fold less than in the D3 model, respectively (**B**, **C**). Seven-fold more WT 4603 endothelial cells produce glucose transporter 1 (GLUT1) compared to D3 cells (**D**, **E**). WT 4603, WT 19-9-7th, WT AD3-CL1 and SMA I endothelial cells express tight-junction protein occludin, whereas D3 cells do not (**F**-**K**). Our *in vitro* models of the BBB with endothelial cells alone display tight junction that closely mimics the human BBB *in vivo* and will have many potential uses including testing of therapeutic agents and investigating BBB breakdown in disease states. We are currently testing the crossing of therapeutics for SMA through our BBB models.

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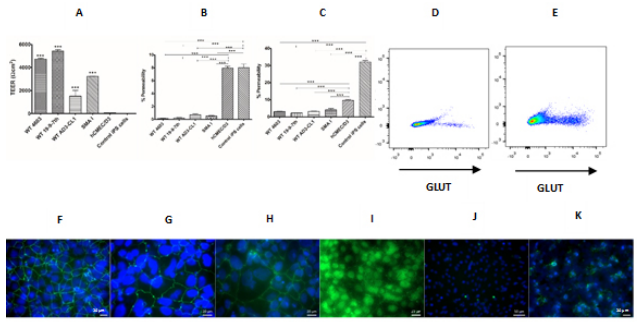

A) TEER of BBB comprised of endothelial cells derived from iPS cells compared to D3 cells and control iPS cells, 48 hours after seeding. B) Permeability of LY through BBB compared to control iPS cells. C) Permeability of NaF through BBB compared to control iPS cells. D) Flow cytometry analysis of D3 cells for detection of GLUT1. E) Flow cytometry analysis of endothelial cells derived from WT-4603 cells for the detection of GLUT1. F-K) Immunocytochemistry for detection of tight-junction protein Occludin by endothelial cells (F, WT 4603, G, WT 19-9-7th, H, WT AD3-CL1, I, SMA I, J, D3 and K, control iPS cells).

155. Application of Cell Sheet Technology for Regenerative Medicine: Therapeutic Angiogenesis and Beyond

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Cell sheets (CS) are a promising technique for cell therapy and regenerative medicine. Their efficacy relies on a number of factors including higher cell survival and engraftment. Cell sheets comprising of viable cells and extracellular matrix (ECM) proteins are feasible due to quick assembly and absence of chemical or xenogenic materials. We previously developed a method to generate CS from adipose-derived mesenchymal stem cells (ADSC) for stimulation of angiogenesis and tissue repair and evaluated them in a model of hind limb ischemia to show that they were superior to dispersed cells injection. We also developed effective methods for viral modification of CS using baculovirus and AAV to express cytokines and growth factors mediating ADSC regenerative effects to see that modified CS show higher therapeutic potential in a number of animal models of ischemia and tissue damage. However, little is known about mechanisms underlying high efficacy of CS delivery and we set on a survey to investigate into this comparing them to standard 2D cultures used for ADSC-based cell therapy products. We hypothesized that CS may have higher paracrine activity compared to monolayer-cultured cells and compared amount of angiogenic growth factors produced by ADSC cultured in either manner. We found that 2 crucial angiogenic and tissue-protective cytokines - VEGF165 and HGF were strongly (up to 5-8-fold) increased in conditioned medium samples from CS compared to monolayer cultures. Another mechanism of CS higher efficacy of increased ECM production and, thus, additional pro-regenerative stimuli within the constructs. Using histological immunoassays we found that CS contain collagens I and III, fibronectin and laminin and these components are abundant within the constructs. Indeed, in CS compared to monolayer cultures quantities of ECM components

were significantly higher in particular due to addition of ascorbic acid as a booster of ADSC proliferation and a known stimulator of collagen production. Attempting to isolate ECM to test its role as a pro-regenerative substance we used decellularization by deoxycholic acid and a number of other detergent compounds to find that decellularized matrix from ADSC-derived CS is mechanically fragile yet can be and manipulated and subject to study. We also found decellularized CS to contain minimal amounts of residual DNA and detected all components of ECM found in CS prior to treatment by detergents. Thus, we may conclude that CS technique may go far beyond using it as a minimal tissue-engineering construct for "patching the tissue". Their therapeutic activity relies on enhanced (even without viral modification) paracrine activity compared to monolayer cultures, increased ECM production and, thus, better ADSC engraftment and influence on surrounding target tissues. Probably CS can also be used as a source of ECM-based biomaterials that may be more feasible due to higher stability compared to living ADSC and lesser immunogenicity for the recipient. *Study was supported by grants from Russian Science Foundation (№16-45-03007) and Ministry of Science and Technology, Taiwan (№MOST 105-2923-E-007-002-MY3)*

156. Treatment of Streptozotocin Induced Diabetes Mellitus in Mice by Subcutaneous Intra Adipose Tissue Transplantation of Beta Cells Induced from Bone Marrow Cells

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The study aimed to measure the ability of IPCs cells induced in vitro to treat diabetic mice in vivo & to compare whether transplanted intra-renal capsule, tip of spleen or inside the Adipose tissue of the neck is the best site for transplantation. Bone marrow- derived mesenchymal stem cells (MSCs) were obtained from Swiss albino mice & propagated in a primary culture & passaged twice before differentiation into islet cells. This was achieved by four-step protocol using high & low glucose MEM, retinoic acid, nicotinamide, epidermal growth factor, fibroblast growth factor & exenden-4. Islets cells differentiation in vitro were followed up by immunocytochemistry, dithizone assay staining, insulin production quantification by ELISA assay and light and scanning Electron Microscope. To determine whether the differentiated BM-MSCs cells possessed the capacity to correct hyperglycemia in diabetic mice, Swiss albino were induced by STZ to become diabetic by single dose (200mg/kg) & occurrence of hyperglycemia. Blood glucose level was elevated above 350mg/dl. The in vivo experimental groups were divided into several groups were differentiated cells transplanted into three different sites, 1- tip of spleen, 2- intra renal capsule & 3- intra adipose tissue, each transplantation site had a control were undifferentiated stem cells were injected. blood glucose levels were monitored three times a week. The results showed differentiation of MSCs into functional IPCs that confirmed by immunocytochemistry, as the IPCs cells were positive for the insulin, c-peptide and glucagon proteins. Moreover, high insulin production by IPCs as quantified by ELISA assay & confirmed by dithizone positive IPCs. The in vivo results showed that transplantation of IPC IPCs cells were successful to treat diabetic mice whether transplanted in intra-renal capsule, tip of spleen or inside the Adipose tissue of the neck. In conclusion, transplantation of the IPCs cells in the adipose tissue of the neck is the best site for treatment as it is less invasive and less stressful on the animals. Furthermore, it is easier and faster & at the same time it causes the same level of treatment that induced by the other sites of transplantation.

157. Development of an Intrinsic Skin Sensor for Blood Glucose Level with CRISPR-Mediated Genome Editing in Epidermal Stem Cells

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Biointegrated sensor can address various challenges in medicine by transmitting a wide variety of biological signals. A tempting possibility that has not been explored before is whether we can take advantage of genome editing technology to transform a small portion of endogenous tissue to an intrinsic and long-lasting sensor for physiological signals. The human skin and the epidermal stem cells of skin have several unique advantages, making them particularly suited for genetic engineering and applications *in vivo*. In this report, we develop a novel platform for manipulation and transplantation of epidermal stem cells, and present the key evidence that genome-edited skin stem cells can be exploited for continuous monitoring of blood glucose level *in vivo.* Additionally, by advanced design of genome editing, we develop autologous skin graft that can sense glucose level and deliver therapeutic proteins for diabetes treatment. Our results reveal the clinical potential for skin somatic gene therapy.

158. Engineering Polymeric Scaffolds to Enhance the Transplant and Efficacy of Neural Stem Cell Therapy for Post-Operative Glioblastoma

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Tumor-homing neural stem cells (NSCs) are a promising delivery vehicle for targeting therapeutic payloads selectively to aggressive brain cancers, including glioblastoma (GBM). First-in-human NSC-mediated enzyme/prodrug phase I clinical studies are ongoing, injecting enzyme-producing NSCs directly into the walls of the GBM resection cavity. However, a major drawback to this administration method is the loss of a percentage of NSCs due to the hostile microenvironment. We recently discovered that bio-compatible scaffolds can stabilize and protect the therapeutic stem cells within

the post-resection tumor cavity, significantly increasing viability. As such, the goal of this study was to develop a new scaffold-based composite implant system capable of promoting efficient NSC delivery and viability to maximize therapeutic efficacy against residual GBM. Using the research equivalent NSC line currently in clinical trials and mouse models of orthotopic GBM resection/recurrence, kinetic imaging showed that delivery on electrospun poly(L-lactic acid) scaffolds extended intra-cavity NSC persistence from 3 to 9 days. We found that varying fiber diameter, three-dimensionality, and coating had minimal impact on NSC persistence. In contrast, gelatin-based matrices (GEM) increased post-transplant levels of NSC 8-fold and prolonged persistence 9-fold compared to direct injection. GEM remained permissive to tumor-tropic homing, as NSCs migrated to tumor foci in 3-D culture models and residual invasive tumor foci *in vivo*. Mirroring human patient testing, NSC/GEM enzyme/pro-drug therapy seeded in the post-operative cavity reduced residual patient-derived GBM volumes 10-fold at 35 days and extended median survival of tumor-bearing mice from 31 to 46 days. Together, these data begin to define design parameters necessary to engineer effective NSC/scaffold composites and suggest a new approach to optimizing the efficacy of NSC therapy in patient trials.

159. NOTCH Signaling Is Essential for Maturation, Self-Renewal and Tri-Differentiation of *In Vitro*-Derived Human Neural Stem Cells

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Neural stem cell (NSC) transplantation is a promising therapeutic strategy for restoring neuronal functional network in various neurodegenerative diseases and spinal cord injuries. Although NSCs have potential applications in treating neurological disorders, much still needs to be understood about the differentiation biology for its successful clinical translation. In this study, we aimed to derive NSCs from human umbilical cord blood derived mesenchymal stem cells (hUCB-MSCs) and explore the role of Notch signaling in the differentiation process. Neurospheres were successfully generated from hUCB-MSCs by induction with epidermal and fibroblast growth factors **(Fig 1-i: e-h &iii:c)** and were enzymatically dissociated using accutase into single-celled NSCs. Clonal analysis of NSCs was carried out through neurosphere-colony forming assay. The expression of NSC markers: Nestin and Musashi-1 were immuno-phenotypically characterized using immuno-fluorescence (IF) **(Fig 1-ii)**. The role of Notch signaling pathway was analyzed using a specific γ-secretase inhibitor known as DAPT. DAPT treatment down-regulated the expression of the NSC marker- nestin at different time points (6h, 12h, 24h, 36h and 5 days) following differentiation from hUCB-MSCs indicating that the process is dependent on Notch signaling. This data was further correlated with formation of reduced average number of neurospheres from hUCB-MSCs as determined through colony-forming assay in the presence of DAPT (2 colonies vs 11colonies/field of view) **(Fig 1-iii: a, b &d)**. To examine the functional properties of hUCB-MSC-derived NSCs its tri-potential (astrocytes, oligodendrocytes and neurons) differentiation capability was tested in presence or absence of DAPT. In presence of DAPT, the markers for neuronal (MAP2, NEFH); and glial (GFAP, GLUL and MBP) lineages were significantly down-regulated as seen via IF indicating the role of Notch in the tri-differentiation mechanism of NSCs as well **(Fig 1-iv)**. These results suggest that the efficient derivation of NSCs and their subsequent lineage commitment from hUCB-MSCs requires Notch signaling pathway. In addition Notch signaling inhibition induced higher cell death during the lineage commitment of NSCs as measured 3 days (16.9% vs 8.9%) **(Fig 1-iii: e-f)** and 5 days (42.9% vs 20.8%) post induction **(Fig 1-iii: g-h)**. Thus, Notch signaling is essential not only for maturation of NSCs from hUCB-MSCs but also for their trilineage differentiation and survival.

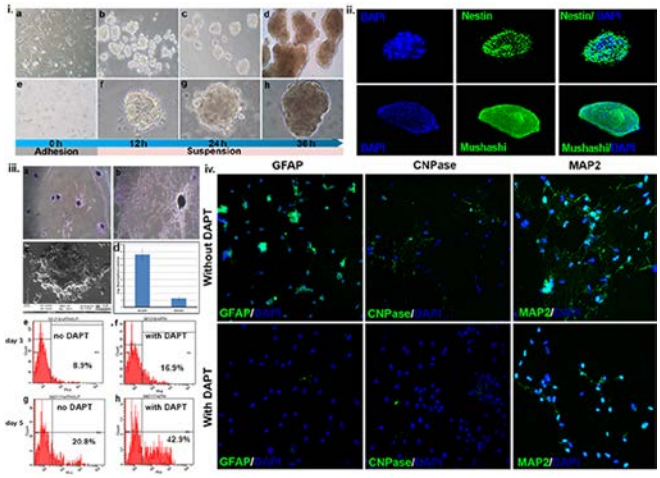

Fig 1: Morphological and functional characterization of MSC-derived neural stem cells and role of Notch signaling in their differentiation. (i) a. MSCs grown in α-MEM with 10%FBS, **b-h.** Schematic of generation of neurospheres from MSCs at different time points, **(ii)** IF images of Nestin and Mushashi positive MSC-derived NSCs, **a.** Only DAPI, **b.** Nestin^{+}/Mushashi^{+} (Alexa Fluor 488), **c.** Merged (a+b), **(iii)** Neurosphere-forming colony assay of NSCs, **a.** without DAPT, **b.** with DAPT, **c.** Scanning electron microscopic image of neurosphere, **d.** Graphical representation of neurosphere colonies formed from MSCs in the presence or absence of DAPT quantified from 13 random fields for each, **e-h.** Flow cytometric analysis of propidium iodide stained cells with and without DAPT 3 days (e-f) and 5 days (g-h) post induction, **(iv)** The expression of tri-lineage markers (GFAP, CNPase and MAP2) was significantly down regulated (Alexa Fluor 488) upon DAPT treatment as observed by IF.

Clinical Protocol Development, Regulatory Interactions, and Ethics

160. Impact of the 2016 Changes to the NIH Guidelines on Registration of Human Gene Transfer Clinical Trials

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The overwhelming majority of human gene transfer clinical trials conducted in or from the United States are subject to the *NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules* (the *NIH Guidelines*). The *NIH Guidelines* are promulgated by the National Institutes of Health (NIH), and compliance with the *NIH Guidelines* is a condition of funding from the NIH. In April 2016, the NIH revised the portion of the *NIH Guidelines* governing the registration process for human gene transfer clinical trials, with the goal of reducing the time and effort needed to navigate the registration process. These changes included a reduction in the amount of information and paperwork associated with the registration process, and a transfer of the initial review process away from the experts on the NIH Recombinant DNA Advisory Committee (RAC) and to local oversight bodies at the institution associated with the initial registration. In short, these local oversight bodies, including the Institutional Review Board and Institutional Biosafety Committee, are now required to assess the registration documents and make a recommendation as to whether or not the protocol would benefit from review by the RAC, prior to the protocol being considered for review by the experts on the RAC. Assessment of the registration records on the NIH's registration portal GeMCRIS indicate that this revised policy resulted in an instant and dramatic decline in the number of human gene transfer clinical trials registered with the NIH. Since January 2013, the NIH had registered an average of 24.5 ±8.5 human gene transfer protocols per quarter. In the three months following the revisions to the *NIH Guidelines*, this number dropped 84% to a total of only 4 protocols. Although the rate of protocol registrations increased over the rest of 2106, the recovery remained incomplete. As a result, 2016 saw a 30% reduction in the number of human gene transfer clinical trials registered with the NIH compared to an average of the past 3 years, and a 45% reduction compared to the previous year. These results are consistent with anecdotal reports of institutions and clinical trial sponsors grappling with the new registration process, and the hesitancy of local oversight bodies to take on new responsibilities once relegated to the experts on the RAC. Updated trends and candidate solutions will be presented.

162. Machine Learning Models for Development of Gene and Cell Therapies

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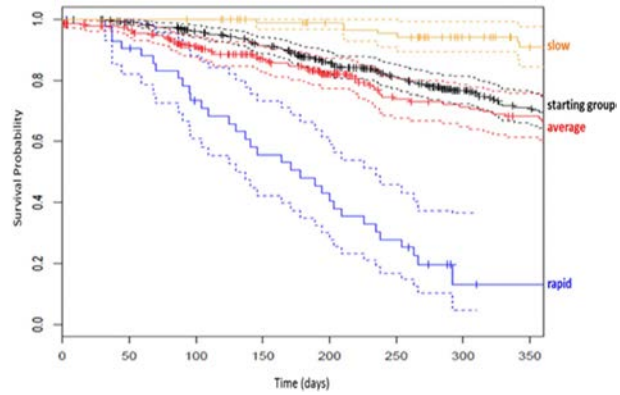
Background: In the absence of placebo groups, clinical trials of cell and gene therapies could benefit from objective models of disease progression. We use amyotrophic lateral sclerosis as a disease to test the use of machine learning models to develop the concept of a virtual control against which an intervention could be compared. The virtual controls are intended to serve as objective measures of efficacy in clinical trials that lack control arms, whereas the stratification tool is envisioned as a tool to identify the best patients for a given intervention. **Objectives:** We hypothesized that computer models incorporating predictions for both survival and disease progression could serve as tools to develop virtual controls and to stratify patients into slowly, average and rapidly progressing patients. **Methods:** We first developed Random Forest (RF) and Gradient Boosting Machine (GBM) models for ALSFRS-R and survival using the appropriate packages in the R programming language. These models were used to develop virtual predicted ALSFRS-R progression and survival virtual control curves that can be compared to observed disease progression and Kaplan-Meier survival curves. For patient stratification, a randomly selected sample from the PRO-ACT ALS clinical trial database was selected as an "industry standard" *in silico* trial population for detailed analysis and the models were trained using the remaining PRO-ACT records. The one year predicted 10% highest mortality patients were defined as rapid progressors while the 25% slowest progressors by predicted ALSFRS-R change were defined as slow progressors. The remaining group was defined as average progressors. We plotted out the actual observed Kaplan-Meier survival curves of the rapidly, average and slowly progressing patients, compared them to each other and to the original starting group of 425 patients. **Results:** The virtual controls and stratification protocols provided accurate representations of the disease progression at the level of individual patients. The predicted slowly progressing group had an observed ALSFRS-R slope of -0.44 pts/month and approximately 90% survived one year. In contrast, the predicted rapidly progressing group had an observed slope of -1.80 pts/month and a median survival of 5.8 months while the average progressing group closely resembled the starting group of 425 patients. **Discussion:** We conclude that virtual controls and patient stratification based on advanced machine learning can provide useful drug development tools for cell and gene therapies.



Gene Targeting and Gene Correction I

163. Towards Clinical Translation of Hematopoietic Stem Cell Gene Editing for the Correction of SCID-X1 Mutations

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The scope of genetic engineering of hematopoietic stem/progenitor cells (HSPC) has broadened from gene replacement to genome editing using artificial nucleases, enabling precise modification of endogenous genes. Yet, we have shown that editing is constrained in long term SCID-repopulating HSPC, likely due to low expression of the Homology Directed Repair (HDR) machinery, cell quiescence and limited uptake of template DNA. By tailoring delivery platforms and exploring culture conditions that induce proliferation while preserving engraftment capacity, we partially overcame these barriers and provide evidence of targeted integration in human HSCs by long-term multilineage repopulation of xeno-transplanted mice. We exploited this strategy to insert a functional cDNA into the *IL2RG* gene, whose mutations cause SCID-X1, showing proof-of-principle of the functionality of an edited *IL2RG* allele in human HSPC. Here, in order to improve the tolerability of the procedure and establish a clinically compatible gene correction protocol we optimized reagents and scaled-up the genome editing procedure. We developed and optimized a new ZFN pair targeting the upstream region of the *IL2RG* gene to correct the majority of SCID-X1 mutations using a single ZFN/donor set. By targeting a corrective cDNA to *IL2RG* intron-1 in primary T cells, we found that targeted cells were functionally indistinguishable from wild-type cells, proving the functionality of the edited gene. To improve nuclease expression while decreasing cellular innate response to mRNA transfection we included modified nucleotides during mRNA production and performed HPLC purification after in-vitro transcription. The use of this optimized mRNA allowed decreasing type-1 interferon activation and significantly improved the editing efficiency. To further optimize ex-vivo HSPC manipulation we tested pyrimidoindole derivatives added to the culture and found a combination promoting HSPC expansion in conditions that preserve their primitive phenotype, increasing the yield of edited cells that are able to repopulate NSG mice. By optimizing dose and timing, we found that AAV6 vector outperforms IDLV for delivering the HDR template, reaching up to 40% targeted integration in bulk treated CD34+ cells and ~13% upon transplant in NSG mice. Deep sequencing performed on treated CD34+ proved the high specificity of our optimized ZFNs, with no significant modification at any of the off-target sites previously identified by GUIDE-Seq for earlier generation ZFNs. By using high volume electroporators, we have now scaled up the manufacturing process and have successfully treated up to 25 million HSPC with highly qualified reagents. Finally, we demonstrated the therapeutic potential of our strategy by correcting the *IL2RG* gene in HSPC from a genotyped SCID-X1 patient. Overall, these studies demonstrated editing of HSPC with efficiency and specificity suitable for clinical translation, thus providing a blueprint for the next generation precise engineering of hematopoiesis for treating SCID-X1.

164. Priming Hematopoietic Stem and Progenitor Cells for CRISPR/Cas9-Mediated Homologous Recombination

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[*]*J.C. and C.T.C contributed equally to this work*

Highly efficient engineered nuclease-mediated gene editing through homologous recombination (HR) in hematopoietic stem and progenitor cells (HSPCs) gives rise to the opportunity of developing gene corrective therapies for hematological disorders. The β-hemoglobinopathies, such as sickle cell disease (SCD) and β-thalassemia, are inherited blood disorders that manifest because of mutations in the beta-globin gene (*HBB*) and afflict millions of people worldwide. The only curative treatment for these blood disorders is allogeneic-hematopoietic stem cell transplant (allo-HSCT) from a healthy donor, which comes with severe limitations, such as a shortage of immunologically matched donors, graft-versus-host disease, and graft rejection. With the lack of effective curative therapies, it is postulated that autologous HSCT of *HBB* gene corrected HSPCs would be a potential one-time curative treatment for patients afflicted with β-hemoglobinopathies without the risk of graft versus host diseases and graft rejection, as well as avoiding the need to find an immunologically matched donor. We have previously described a methodology for targeting the *HBB* gene through HR using the CRISPR-Cas9 system, delivered as a Cas9 protein precomplexed with chemically modified sgRNAs along with rAAV6 transduction to deliver the homologous *HBB* gene corrective DNA template. Building upon this work, we have further optimized 1) CRISPR-Cas9 nuclease delivery, 2) AAV6 DNA donor delivery and 3) HSPC cell culture conditions, which altogether have resulted in reproducibly more efficient *HBB* gene targeting in HSPCs. Genome edited HPSCs displayed long-term engraftment in the bone marrow of immunodeficient NSG mice at 16 weeks post-secondary transplant where we identified that ~49% of human cells had INDELS in the *HBB* gene, matching our input, implying the CRISPR/Cas9 system is effective at inducing double strand breaks at the *HBB* locus in long term repopulating stem cells. Additionally, electroporation aided AAV6 transduction (EAAT) revealed a ~2-fold increase in transduction using scAAV6 compared to non-electroporated conditions. Furthermore, we have also found that HSPCs cultured with cytokines at low densities (1E5-4E5/ml) for ~48 hours stimulates cell cycling, priming HSPCs for HR using EAAT, and more importantly, we were able to demonstrate that cell growth and rates of HR strongly positively correlated using linear regression analysis ($R^2 \approx .6$). In conclusion, upon revisions to the delivery of the CRISPR/Cas9 nuclease, rAAV6 transduction, and culture conditions of HSPCs, we have not only shown that HR rates can be significantly improved, but such rates can be consistently reproduced, which is critical to its use as a therapeutic treatment.

165. A Novel Gene Therapy Approach of Fanconi Anemia Hematopoietic Stem Cells Based on NHEJ-Mediated Gene Editing

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Introduction: Gene editing constitutes a new step in the development of safe gene therapy approaches for patients with inherited diseases such as Fanconi Anemia (FA). Previous results from our group have demonstrated the feasibility to correct FA patients' hematopoietic stem and progenitor cells (HSPCs) by homologous recombination (HR)-mediated gene editing using Zinc Finger nucleases and donor therapeutic constructs. Considering that NHEJ is the preferential double strand break (DSB) DNA repair mechanism in HSPCs and given that this pathway has been reported to be increased in FA HSPCs, we have now aimed at developing a NHEJ-mediated gene editing strategy in HSPCs from FA-A patients. Although NHEJ is an error-prone DNA repair mechanism, its inaccuracy can be exploited in FA to introduce compensatory mutations capable of restoring the function of mutated proteins, mimicking reversions observed in mosaic FA patients.

Objectives: We aimed at demonstrating the feasibility of using a NHEJ-based gene editing approach to correct the phenotype of lymphoblastic cell lines (LCLs) and HSPCs carrying the biallelic c.295C>T mutation, the most frequent mutation reported in FA patients from Spain, that generates a premature stop codon in the fourth exon of *FANCA*, thus leading to a nonfunctional truncated FA protein (p.Q99X). In particular, our goal was to remove the stop codon generated by the c295C<T mutation as a result of the INDELS generated by CRISPR/Cas9 nucleases.

Results: Targeting efficiencies around 20% were achieved with CRISPR/Cas9 and guide RNAs designed for targeting sequences surrounding the pathogenic mutation. Moreover, next generation sequencing (NGS) revealed that frame-restoring repair events took place after gene targeting. Strikingly, gene edited FA clones showed both a marked *in vitro* proliferative advantage over uncorrected cells and also a significant reversion of the characteristic MMC hypersensitivity of FA cells. Western-blot analysis of frame-restored clones demonstrated the stable expression of FANCA. Additionally, evident FANCD2 foci were observed in these cells, confirming the phenotypic reversion of FA cells. Recent studies in primary $CD34^+$ cells from FA-A patients harboring the same mutation have shown targeting efficacies of up to 36%. Moreover, INDELS identified by NGS at different time points after CRISPR/Ca9 nucleofection not only demonstrated the presence of corrective NHEJ-repair events, but also an evident expansion of corrected *vs* uncorrected clones (up to 50 fold) over a 9 day incubation period, indicating the functionality of the frame-restored alleles.

Conclusions: Our results demonstrate the feasibility of conducting NHEJ-mediated gene correction in FA HSPCs. The high efficacy of the NHEJ DNA repair pathway in these cells, together with the simplicity of the proposed strategy, make this approach a clinically relevant strategy to be considered for the future treatment of FA patients.

166. In Vivo Genome Editing with a Small Cas9 Orthologue Derived from Campylobacter Jejuni

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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 mouse muscle cells or 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

167. CRISPR-Cas9-Mediated *In Vivo* Susbtrate Reduction Therapy Rescues Phenotype of Primary Hyperoxaluria Type I

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Primary Hyperoxaluria type 1 (PH1) is a rare, autosomal recessive metabolic disorder of the glyoxylate metabolism characterized by

deficiencies of the hepatic alanine-glyoxylate aminotransferase (AGT). Defective AGT results in excessive oxalate production leading to calcium oxalate stone formation, which results in progressive deterioration of renal function and, eventually, end-stage renal disease. Combined liver-kidney transplantation is the only curative treatment but associated with significant morbidity and mortality. Thus, there is a clear and urgent need for a new safe therapy for hyperoxaluria patients. Substrate reduction therapies (SRTs) aim to prevent the production of oxalate precursors like glyoxylate. Inhibition of glycolate oxidase (GO) by siRNAs in a PH1 animal model has shown to trigger reduction of oxalate levels in urine and to protect the animals against kidney damage. However, siRNA-mediated GO inhibition is transient requiring multiples doses for an efficient and long term effect. Previous studies demonstrated that *in vivo* CRISPR-Cas9 mediated gene disruption is effective when using AAV vectors as carriers of these systems. Moreover, CRISPR-Cas9 mediated modifications are permanent allowing a lifetime effect. Therefore, we developed SRT for PH1 using AAV8 vectors expressing *Staphylococcus aureus* Cas9 (SaCas9) and guide RNAs (gRNA) against *Hao1* exonic regions, which encodes GO enzyme. Our results demonstrated *Hao1* gene disruption in the livers of PH1 animals, revealed by the presence of insertions and deletions (indels) in the regions targeted by gRNA. Moreover, WB and IHC analysis showed a dramatic reduction of GO protein expression. In addition, we observed reduced urine oxalate levels in treated animals after ethylene glycol challenge (a glyoxylate precursor) as well as reduced kidney damage. Currently, the long-term efficacy is being analyzed and next generation sequencing is being performed to evaluate gRNA off-targets. In conclusion, our work demonstrates that an effective GO knock-down was obtained in the liver of animals treated with an AAV8 expressing SaCas9 and specific gRNAs. In addition, effective reduction of oxalate levels and associated toxicity was achieved.

168. Targeted Homologous Recombination within the *WAS* Locus in Human Hematopoietic Stem Cells

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Gene editing in primary human hematopoietic stem cells is a promising technology for achieving curative therapies of genetic diseases affecting the blood and immune system. Wiskott-Aldrich Syndrome (WAS) is a primary immunodeficiency caused by mutations in *WAS*, a gene expressed in virtually all hematopoietic lineages. Besides immunodeficiency, WAS patients have severe thrombocytopenia, eczema and are at high risk for autoimmunity and lymphoid malignancies. A previous gene therapy trial using a γ-retrovirus successfully corrected many of the functional defects in WAS, but nearly all patients subsequently developed leukemia resulting from insertional mutagenesis. With the goal of developing a gene editing therapy for WAS that would minimize the risk of mutagenesis by targeting integration of a therapeutic cassette into the *WAS* locus, we designed an AAV donor template for integrating an expression cassette into the first exon of *WAS* by homology directed repair. In parallel, we established nuclease platforms targeting the *WAS* locus: transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9. Next, using adult mobilized CD34+ cells and co-delivery of either TALEN mRNA or Cas9/gRNA ribonucleoprotein complexes (RNPs) and an AAV donor for targeted integration of a promoter-driven fluorescent marker, we have achieved efficient homology directed repair rates across multiple donors (25-40% for TALEN, and >40% for RNP) with the highest levels of cell viability observed using RNP/AAV co-delivery. Edited HSC retained their potential to give rise to multiple lineages in colony forming unit assays and experiments to assess long-term engraftment and differentiation potential in immune-deficient mice are underway. We are currently evaluating AAV vectors carrying WAS cDNA to restore expression in WAS deficient cells with the goal of achieving therapeutic correction of the disease in patients.

169. Impact of Target Cell Gender and X-Inactivation on Efficiency of Gene Editing with CRISPR/Cas9

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Efficient gene editing by CRISPR/Cas9 delivered via ribonucleotide protein (RNP) complexes of Cas9 protein and specific guide RNAs (gRNA) has many promising applications in disease modeling, gene therapies and regenerative medicine. In the context of developing a rhesus macaque non-human primate model of the hematopoietic stem and progenitor cell (HSPC) disorder paroxysmal nocturnal hemoglobinuria (PNH) caused by acquired somatic mutations inactivating the X-linked PIG-A gene, we investigated whether the presence of two copies of the X chromosome, and the phenomenon of inactivation of X chromosome in female versus male cells would impact on gene editing efficiency of CRISPR/Cas9 targets on the X chromosome, and generation of functional phenotypes.

We analyzed the efficiency of non-homologous end joining editing mediated by CRISPR/Cas9 at the X chromosome PIG-A locus and at the control autosomal locus AAVS1 in male versus female target cells. Two PigA gRNAs targeting PIG-A gene exon 2 were selected from 14 gRNA candidates based on efficiency of editing as screened in female rhesus kidney cell lines (FRhK4) and rhesus mobilized CD34+ HSPC (rhCD34+). We noted reproducibly lower editing efficiency of the PIG-A compared to AAVS1 even in the FRhK4 cell line, but via sustained expression of Cas9 and a PIG-A gRNA from a lentiviral vector, we observed up to 50% insertions and deletions (INDEL) efficiency as tracked by the TIDE assay, resulting in more than 90% of cells acquiring the PNH phenotype characterized by loss of expression of glycophosphatidylinositol (GPI)-linked proteins. The PIG-A gene is located in heterochromatin at Xp22, versus the AAVS1 site localized within autosomal euchromatin. With transient expression of Cas9 and gRNA via RNP electroporation, mean editing efficiencies for multiple targets within the PIG-A gene versus the AAVS1 gene, were up 7.5- 8.1% versus 12.7% (n=3). For rhCD34+ HSPC target cells following RNP transfection, INDELs were up to 9.2% in male cells and 3.5% with female cells, with a mean of 5.5% and 2.6% following a single transfection and editing assessment for

2 weeks of culture. In contrast, the INDEL efficiency for the AAVS1 site in rhCD34+ cells was approximately twice as high with a mean of 11.6% (n=5). When PIG-A gene targeting RNPs were transfected into human female and male B-lymphoblastoid cell lines (LCL), the mean INDEL efficiency was also higher in male cells compared to female cells, 23.2% versus 16.6% (n=5) respectively. When edited female LCL cells with a PNH phenotype were sorted via loss of expression of GPI-linked proteins using FLAER, the INDEL efficiency in the pure PNH population was 48.7%, not 100%, suggesting editing of only the active X chromosome PIG-A allele. In conclusion, we have demonstrated gene editing efficiency for the PIG-A gene within heterochromatin is lower than editing of the AAVS1 locus within euchromatin area, and provide evidence that editing of the PIG-A allele on active versus inactive X chromosomes is more efficient, potentially due to poor accessibility of inactivated loci to gene editing complexes. This results in female XX cells with a lower efficiency of editing compared to XY cells, but equivalent functional editing levels. Our findings imply that gene editing occurs preferentially on the active X chromosome in female cells, and X-linked genes have no disadvantage in gene editing by CRISPR/Cas9 RNP method. We will present data extending our findings to other X-linked loci.

170. A New, Reversed Zinc-Finger Nuclease Structure for High-Precision Therapeutic Genome Engineering

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Genome editing with engineered nucleases has shown broad utility for basic research across the fields of biology, biotechnology, and medicine. Therapeutic applications, however, require cleavage that is not only highly efficient but often targeted to a specific base. To this end, we have used a bacterial selection system to develop new zinc-finger nucleases (ZFNs) in which the Fok-ZFP domain order has been reversed relative to canonical ZFNs (i.e. FokI is now linked to the amino terminus of the designed ZFP). ZFNs bearing the N-terminal FokI can be dimerized with either each other or canonical ZFNs to yield highly-efficient cleavage of their genome targets. The availability of these new architectures increases the targeting capabilities of the ZFN platform by at least a factor of four, and allows fine-scale positioning of a ZFN-induced cleavage event on average at less than every second base in a given sequence target. This enables rapid development of nucleases with precise targeting, high levels of genome modification, and high specificity for therapeutic purposes.

In initial applications, we have used these new architectures to rapidly generate highly-active ZFNs (>85% indels) that cleave at the precise location of the blindness-causing LCA10 point mutation. This mutation, which is located within an intron, disrupts gene function via an unusual mechanism involving activation of an otherwise cryptic splice donor element. It has been proposed that simple cleavage of this location followed by error-prone NHEJ repair could be sufficient to disrupt aberrant splicing and restore gene function, however efficient and precise targeting of this base has thus far proven refractory to other nuclease platforms such as CRISPR/Cas9. The high design densities afforded by the new architectures have also enabled rapid identification of highly-specific and highly-active ZFNs for functional knockout applications, which have larger target windows. In studies relevant to adoptive T-cell therapy, we have rapidly generated a diversity of ZFNs that efficiently disrupt both beta-2 microglobulin and the T-cell receptor alpha constant region. Critically, these studies were performed at large scale and used high ZFN levels for both the capture and follow-up assays (e.g. >80% on-target modification in T-cell studies). Assessment of these via unbiased genome-wide specificity analysis using an oligonucleotide duplex capture assay, followed by screening of candidate off-target sites for modification in ZFN-treated T-cells, identified a highly-active pair for each target that exhibited little or no detectable off-target cleavage.

171. High-Fidelity Genome Editing of Therapeutic CAR-T Cells Using a Novel Clo51-dCas9 (NextGEN™) CRISPR System

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The CRISPR/Cas9 system has ignited a new era for genome editing but its clinical applications have been hampered by the naturally high off-target activity, which may result in unwanted and potentially detrimental mutations, such as the activation of proto-oncogenes or disruption of tumor suppressor genes. The significant risks associated with transforming genetic modifications are widely regarded to be unacceptable in the clinical application of gene-edited cells. Here we report that this undesirable limitation is surmounted by using a novel Clo51-dCas9 genome editing technology (NextGEN™ CRISPR). This RNA-guided DNA endonuclease retains the efficiency and ease of use of CRISPR/Cas9, while eliminating or dramatically reducing the off-target activity by incorporating the exquisite site-specificity of a type IIS restriction endonuclease. Distinct from the single gRNA guided CRISPR/Cas9, the Clo51-dCas9 system uses a dimeric gRNA-guided nuclease, in which each half-site subunit contains a fusion protein of a catalytically inactive Cas9 (dCas9) and the novel type IIS restriction endonuclease Clo51. Like FokI, which has been extensively used in the applications of TALEN and zinc figure nucleases, Clo51 activity is contingent upon formation of a Clo51 homodimer, and thus DNA cleavage is strictly dependent on the simultaneous on-target binding of two gRNA-guided endonucleases; individually each half-site is unable to nick or cut DNA. We tested the efficiency of the NextGEN™ CRISPR system by targeting several key surface-expressed cellular markers known to be critical in mediating cellular graft response. Transplant of allogeneic T cells can mediate graft-vs-host disease (GvHD) through the T cell receptor (TCR), which may lead to patient organ damage and lethality. In addition, donor cells can be rejected by the patient through recognition of donor major histocompatibility complex I (MHCI), potentially limiting the therapeutic effect. Thus, we designed a number of guide RNA (gRNA) pairs specific for the human T cell receptor α chain (TCRα), β chain (TCRβ) and β-2 microglobulin (β2M), and assessed their genome targeting capacities using the

NextGEN™ CRISPR system. We measured cutting efficiencies in both activated and resting T cells, the latter posing the greatest challenge due to more compact chromatin. Lastly, we investigated off-target cutting by either NextGEN™ or wild type (WT) CRISPR reagents in a side-by-side comparison using identical gRNAs. We report high gene disruption efficiencies in both activated and resting T cells using NextGEN™ CRISPR. Efficiencies of 84% knockout for surface TCRα, 91% for TCRβ and 62% for β2M were achieved in resting T cells. These data demonstrate that the NextGEN™ CRIPSR system is highly efficient, even in predominantly non-dividing cells. In contrast to the high cutting efficiency observed when both half-site gRNAs were delivered, no on-target disruption was observed with either half-site reagent alone. In addition, results from a side-by-side comparison of WT and NextGEN™ CRISPR indicate that, at the same gene locus, the NextGEN™ CRIPSR system targets the genome as efficiently as WT, but without detectable off-target mutations as measured by deep-sequencing. NextGEN™ CRISPR is an effective technology for genome editing of human T cells for therapeutic uses and is safer than WT CRISPR because of its eliminated or greatly reduced off-target cutting. Capable of knocking out specific genes with high efficiency in resting T cells, it can be applied to the manufacture of allogeneic T cell therapies to reduce the incidence of GvHD and graft rejection. The capabilities of editing genomic DNA in non-dividing cells with high fidelity provides more flexibilities for gene modifications during clinical applications.

172. Evaluation of On-Target and Off-Target Precision of AAVHSC-Mediated Genome Editing

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Variation within the human genome underlie a vast spectrum of diseases. The discovery of genome editing technologies now provide the potential to correct pathogenic mutations and bring the possibility to cure genetic diseases. However, the development of effective and safe therapeutic editing technologies will require highly sensitive assays to identify and quantitate the accuracy of genome editing. Thus it is critical to develop unbiased methodologies to identify i) on-target editing errors, including insertions/deletions (indels) that are commonly seen during non-homologous end joining and ii) unintended off-target mutations, including random viral integration. Adeno-associated virus (AAV) vectors are proven nonpathogenic gene therapeutic tools. AAVHSCs (AAV-Hematopoetic Stem Cell derived), a novel, naturally-occurring family of AAVs mediate highly efficient (HR) homolgous recombination-based genome editing (Smith et al ASGCT 2017). Here we measure AAVHSC HR mediated genome editing by targeted insertion of a promoterless fluorescent reporter gene into Intron 1 of the PPP1R12C gene on Chromosome 19 in CD34+ primary human hematopoetic stem cells. Editing was measured using fluorescence expression in parallel with quantitative genotyping by edit-specific droplet digital PCR and next generation sequencing (NGS) of the target site. Analysis of on-target NGS reveal high precision of AAVHSC editing, with an on-target indel rate of less than $2.63e^{-6}$ indels/cell, comparable to the error rate of Taq polymerase. Additionally, there was no sequence evidence of incorporation of viral DNA elements including inverted terminal repeats (ITR). To identify off-target genome alterations, we developed non-hypothesis driven approaches which use the vector genome as bait to capture all sequences in proximity to the vector thus identifying both on-target and off-target genomic integration events. Analysis of whole genome NGS mapping data revealed that 99.972% of captured sequences perfectly map to the genomic target, whereas off-target reads were observed at frequencies comparable or lower than the expected rate of AAV integration, (609 of 2,211,588 reads, $<1.25e^{-4}$ events / cell). Further characterization of off-target insertion revealed that repetitive elements, when included in the vector correlate with off-target insertion events into similar repetitive elements, thus providing valuable insights for improved vector design. Lastly, we have employed these genome editing analytical tools to the development of therapeutic AAVHSC genome editing vectors for the correction of genetic disease. In conclusion, we have developed and employed novel genome editing characterization tools and show that AAVHSC-mediated genome editing is highly precise for on-target editing and is accompanied by rare off target integration events. Thus, this evaluation of AAVHSC-mediated editing provides a path toward novel genome editing therapeutics for the treatment of human genetic diseases.

173. Utilizing Human Whole Blood to Predict *In Vivo* Immune Responses in Human Setup Against *In Vitro* Transcribed Chemically Modified CAS9 mRNA

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Objective: mRNA based therapy is becoming an attractive tool for treatment of single gene disorders, either as a gene supplementation tool or a gene editing tool to correct genomic aberration. However, *in vitro* transcribed unmodified mRNA possesses the risk of triggering innate immune responses due to detection by the innate immune system. Combinations of chemically modified nucleosides has been shown to reduce such responses, but an assay reflecting the complexity of the human immune system and its reaction upon mRNA transfection is still needed. Here we present a method to easily screen mRNAs for cytokine responses from the human immune systems.

Method: A range of chemically and codon optimized *Cas9* mRNAs, with or without HPLC purification, were co-applied together with TransIT (a lipid-based RNA transfection reagent) to whole blood, which was collected from three healthy human donors. Serum was collected after 6 and 24 hours, respectively, followed by cytokine detection of IL-12, TNF-α and INF-α with human specific ELISAs. On

the basis of the whole blood assay a number of chemically modified *Cas9* mRNAs were chosen for i.v. (intravenous) injection into mice (n=3). Blood collection was performed at 6 hours and 24 hours after injection respectively, and examined for immune response as described above. Furthermore, THP1- Dual cells (derived from human THP-1 monocytes) were investigated to evaluate general IFN response against *Cas9* mRNA by assessing the activity of Lucia luciferase as readout of activating an IFN responsive promoter.

Results: Sequence optimization reduced immune response drastically, and often under the detection limit. Unmodified *Cas9* mRNA, which actually induced strong immune responses *in vivo*, shows the same high reaction in the presented whole blood assay and the THP-1 system. Intriguingly, HPLC purification seems to play a minor role in reducing immune responses with some modifications, observed both by whole blood assay and THP1-Dual cell system. However, it certainly will play a role for the clinical trails where the therapeutic compound has to be clearly specified, removing any unwanted debris.

Conclusion: The study shows that a whole blood assay can be used to mimic human immune responses against *in vitro* transcribed mRNA. Intriguingly, sequence optimization, in combination with some base modifications, may reduce the need for HPLC purification.

174. Adenoviral Vectors Delivering HPV Oncogene Specific CRISPR/Cas9 for Treatment of HPV Related Cancers

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Persistent infections with human Papillomavirus (HPV) cause cervical cancer. HPVs are also involved in the development of anogenital, head and neck as well as non-melanoma skin cancer emphasizing the role of HPVs as major carcinogenic agents. Mainly the HPV oncogenes E6 and E7 drive transformation of infected cells by destruction of the tumor suppressor (p53) and inhibition of the retinoblastoma protein (pRB) resulting in inhibition of apoptosis and induction of cell cycle progression. It has been shown that designer nucleases such as TALEN and CRISPR/Cas9 targeting HPV oncogenes show high target DNA disruption efficiency, which leads to decreased inhibition of p53 and pRB. In cervical cancer cells that are characterized by HPV oncogene mediated inhibition of apoptosis and cell cycle induction, nuclease treatment leads to cell cycle arrest and increased cell death. However no attempts were made to improve designer nuclease treatment for HPV related cancers by means of viral delivery allowing for translating these promising findings towards *in vivo* applications. Here we aimed at arming gene deleted high-capacity adenoviral vectors (HCAdVs) as well as E1/E3 deleted early generation adenoviral vectors (AdV) with the HPV-E6 specific CRISPR/cas9 machinery. By using a new toolbox that facilitates customization, cloning and production of CRISPR-HCAdVs we assembled HCAdV and E1/E3 deleted AdV genomes containing the Cas9 (spCas9) gene together with either one gRNA expression unit specific for HPV18-E6 or two gRNA expression units specific for HPV18-E6 and HPV16-E6. HPV specific CRISPR-HCAdVs and E1/E3 deleted CRISPR-AdV were amplified and purified to high titers. Hela and Caski cervical cancer cells containing HPV18 or HPV16 genomes integrated into their cellular genome, as well as HPV negative A549 cells were infected with HPV specific CRISPR-HCAdVs or E1/E3 deleted CRISPR-AdV. Adenoviral delivery of HPV specific CRISPR/Cas9 resulted in strong cell death in HPV positive cervical cancer cell lines whereas HPV negative A549 cells were unaffected. Moreover, HPV-specific CRISPR-HCAdVs and E1/E3 deleted CRISPR-AdV infected Hela and Caski cells showed increased apoptosis induction and decreased proliferation and viability compared to untreated cells and HPV negative control cells. Our results suggest that CRISPR-E1/E3 deleted AdV and also HCAdVs can serve as oncolytic agents when armed with target specific designer nucleases such as CRISPR/Cas9. We believe that our approach will pave the way towards *in vivo* applications of CRISPR/Cas9 mediated oncolysis of HPV induced cervical cancer. 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 antiviral or oncolytic vectors for the treatment of HPV related cancer depending on the HPV type present in the respective tumors of a patient.

175. Proof of Concept - CRISPR-Cas9 Lipid Nanoparticles as an Efficient Delivery Tool for Cultured Cells and in Animal Models

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Advances in the gene editing arena, specifically with CRISPR-Cas9, has pushed the demand for efficiently delivering payloads even further. Of the tools available, developments in the field of lipid nanoparticles (LNPs) has allowed for the reliable and efficient delivery of CRISPR components, both in research and clinical settings. Here, we bridge that gap by describing the development of an LNP delivery system for CRISPR components, robustly manufactured with clinical-grade materials using microfluidic technology at scales for screening applications, *in vitro* experiments and research in animals. We describe the use of lipid-based nanoparticles for highly efficient encapsulation and delivery of payloads, such as siRNA, mRNA and plasmid. In this proof of concept, we show that representative small RNAs, mRNAs and plasmids can be successfully delivered to primary neurons. LNPs manufactured to encapsulate various nucleic acids can do so with high efficiency, encapulating more than 95% of the payload, minimizing payload loss. Transfection efficiency of the LNPs is >95%, quantified using a flourescent dye. The biological endpoint assays used to determine the accessiblility of the payloads delivered varies for siRNA, mRNA and plasmid. Using doses of 1μg per mL of media, we achieved >90% knockdown with siRNA delivery, >90% of the primary neurons are GFP+ with GFP mRNA delivery and >60% of the primary neurons are GFP+ with GFP plasmid delivery. The LNPs are well tolerated,

such that 5x the required doses have no oberservable cytoxicity. We show that the LNPs can also be used to deliver payloads into various regions of the animal brain. The localized injections into the cortex and the striatum are well tolerated and have extensive distribution. These validation studies provide suitable insights in establishing strategies for efficiently delivering CRISPR components into primary cultures and into the animal. The use of LNPs can be extrapolated to CRISPR components with a simple change in payload. We have editing efficiencies associated with delivering gRNAs to Cas9-expressing cells, as well as simultaneously delivering Cas9 mRNA and gRNAs to cells.

176. Genome-Wide Analysis of Talen® Activity in Primary Cells

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Genome engineering with programmable nucleases such as CRISPR-Cas or TALEN® has revolutionized biological research and has broad-based therapeutic applications. However, for clinical use, it is essential to understand the totality of genome-modifying effects of these nucleases as deleterious off-target mutations may create cells with oncogenic potential or impaired function. Genome-wide, unbiased identification of double-stranded breaks (DSBs) enabled by sequencing (GUIDE-seq) is an unbiased approach to identify the on- and off-target effects of programmable nucleases as it marks DSBs as they occur in living cells by the integration of a small, blunt double-stranded oligodeoxynucleotide (dsODN). These labeled DSBs are then amplified by PCR and mapped by deep sequencing. GUIDE-seq was developed using CRISPR-Cas nuclease which creates blunt-ended DSBs and it was unknown if the procedure could be adapted to assess the genome-modifying effects of nucleases such as TALEN® that create 5' overhangs. Furthermore, GUIDE-seq is routinely applied in cell lines which may not accurately reflect the genome-modifying potential of programmable nucleases due to differences in repair mechanisms inherent to primary cells and immortalized cell lines. Here we adapted the GUIDE-seq procedure to measure the genome-modifying effects of TALEN® in primary human T cells. We show that the procedure is highly sensitive as combining samples treated with TALEN® with non-treated samples allowed us to define the percentage of TALEN®-treated cells necessary in the sample to identify the on-target effects of the TALEN®. In addition, the procedure is transferrable as the genome-modifying effects of multiple TALEN® were characterized by GUIDE-seq in primary human T cells. In conclusion, GUIDE-seq can be used to assay the genome-modifying effects of TALEN® in primary cells. These results suggest that the procedure is widely applicable to measuring the genome-wide specificities of programmable nucleases and can be readily applied to primary human cells permitting off-target identification in the actual edited cell type.

177. 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 recently exploited the vector's ability to induce homologous recombination (HR) to design promoterless vectors that utilize chromosomal homology arms flanking a ribosomal-skipping P2A and therapeutic coding sequence to integrate sequences just upstream of the stop codon of an endogenous gene. When targeting the albumin gene in the liver, a chimeric mRNA transcript capable of producing both albumin and a second therapeutic protein is consequently created. Not only do these vectors 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, 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 the transcriptional rate and/or chromosomal position effects influence this type of HR.

To do this, we developed a high-throughput strategy to quantify precision AAV-mediated transgene integration by exploiting an engineered locus whose transcriptional rates could be 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 co-expressing luciferase and GFP by linkage with a P2A. The population is subsequently infected with an AAV serotype DJ vector designed to integrate a codon-diversified mCherry and unique barcode that would allow for multicistronic expression of luciferase and mCherry, and simultaneous loss of GFP expression, only if HR occurred (Fig. 1). Our approach of using a drug-inducible, lentiviral-rAAV targeting system was validated in mammalian cells. However, to make it a truly high-throughput approach, we added a genetic barcode that is detectable in both the genomic DNA and in RNA transcripts originating from the doxycycline-inducible promoter. Upon modulating the rate of transcription just prior to rAAV vector administration, we will use the barcodes to (a) identify genomic loci at which HR preferentially occurred for mapping onto existing chromatin state maps, and (b) quantify the level of expression from each doxycycline-inducible genomic locus and correlate it with AAV-mediated HR at that locus. 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.

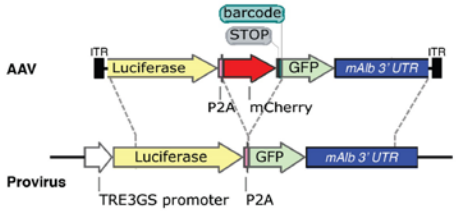

Figure 1. Targeting scheme showing proviral target site and targeting rAAV. Black lines denote host genome.

178. A Gene Deleted High-Capacity Adenoviral Vector for Efficient Delivery of a Multiplex DMD Specific CRISPR/Cas9 Machinery

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Recent advances in the field of designer nuclease directed genome editing hold great promise to correct underlying mutations leading to Duchenne muscular dystrophy. Especially the CRISPR/Cas9 system provides a convenient way to design and to assemble RNA guided nucleases offering the potential to develop personalized treatments to correct the multiple different mutations underlying DMD. Recent studies showed efficient genome editing in a myoblast cell line derived from DMD patients, in mdx mice, and in transgenic mice expressing a mutated version of human DMD following AAV delivery of the DMD specific CRISPR/Cas9 machinery. Nevertheless viral delivery of all required CRISPR/Cas9 components including Cas9 together with multiple guide RNA (gRNA) expression units has not been fully exploited. Gene deleted high-capacity adenoviral vectors (HCAdVs) offer the packaging capacity to deliver the complete CRISPR/Cas9 machinery including several gRNA expression units using a single viral vector. By using a new toolbox that facilitates customization, cloning and production of CRISPR-HCAdVs we assembled a HCAdV genome containing a *Streptococcus pyogenes* Cas9 (spCas9) gene including two guide RNA (gRNA) expression units specific for DMD, that have shown efficiency to delete exon 51 in dystrophic human myoblasts. DMD specific CRISPR-HCAdV was amplified and purified to high titers. Infection of cultured human skeletal myoblast with purified DMD specific CRISPR-HCAdV at different MOIs resulted in strong locus specific deletion efficiency for DMD exon 51 as shown on DNA and mRNA levels as analyzed by locus specific PCR. Our results show that CRISPR-HCAdVs are efficient delivery vehicles for CRISPR based DMD gene editing. Our delivery approach broadens the opportunities for potential *in vivo* applications of CRISPR/Cas9 mediated gene editing for DMD treatment including preclinical and eventually clinical 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 vectors for the treatment for DMD depending on the underlying mutation for every single patient.

179. CRISPR/Cas9-Mediated Editing of Trinucleotide Repeat Expansion in Myotonic Dystrophy

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CRISPR/Cas9 is an attractive platform to potentially correct dominant genetic diseases by gene editing, a concept which remains largely unproven. In the current proof-of-principle study, we explored the use of CRISPR/Cas9 for gene editing in myotonic dystrophy type 1 (DM1), an autosomal dominant disorder associated with severe myotonia and skeletal muscle dysfunction. The DM1 pathology is caused by trinucleotide CTG repeat expansion in the 3' untranslated region (UTR) of the human myotonic dystrophy protein kinase (DMPK) gene. We designed a CRISPR/Cas9-based strategy using dual guide RNAs and S. pyogenes Cas9 that specifically excises this pathogenic CTG repeat expansion in the DMPK 3' UTR. We first generated DM1 patient-specific iPSCs and subsequently induced them to differentiate into myogenic cells and myotubes. One of the hallmarks of DM1 is the emergence of ribonuclear foci that accumulate in the nucleus of patient's cells. CRISPR/Cas9-mediated excision of the triplet repeats expansion resulted in the disappearance of these ribonuclear foci that sequester MBNL1 splicing factors in the DM1-iPSC-derived myogenic cells, resulting in the normalization of the splicing pattern. This proof-of-concept study validates the use of CRISPR/Cas9 to genetically correct nucleotide repeat expansions associated with dominant genetic disorders that cause severe human pathologies.

180. Functional Gene Correction of Cystic Fibrosis Using *In Vitro* Transcribed *Cas9* mRNA

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Objectives: Cystic fibrosis (CF), the most common monogenic disease in Caucasians, is caused by mutations in the encoding *CFTR* gene. The advancement in CRISPR based gene correction technology and *in vitro* transcribed (IVT) mRNA provides the potential to correct the CF disease-causing mutations[1,2]. In this study, we evaluated the functional gene correction efficiency of IVT *Cas9* mRNA together with oligonucleotide repair template against the common CF mutation ΔF508.

Methods: We screened different single guide RNAs (sgRNAs) that were in close proximity to the ΔF508 mutation in the h*CFTR* gene locus. CFBE41o- (ΔF508/ΔF508) cells were utilized to assess gene-targeting efficiency of differently chemically modified *Cas9* mRNAs (cmRNAs) and codon optimized *Cas9* mRNA. The Cas9 expression plasmid JDS246 was used as a pDNA control. Single stranded oligodinucleotides (ssODN) were specifically designed and tested for their ability to create homologous directed repair (HDR) of ΔF508. The corrected cells were tested for CFTR expression by Western blot and for function by a halide sensitive YFP assay. In addition, immunogenicity of IVT *Cas9* mRNA was measured by an *ex vivo* whole blood immune assay.

Results: Compared to pDNA encoded Cas9, the delivery of *Cas9* mRNA resulted in higher indel frequencies ($P < 0.05$) and significantly better gene correction ($P < 0.05$) *in vitro*. Among different *Cas9* mRNAs, codon optimized *Cas9* mRNA was superior in terms of indel formation ($P < 0.001$). The ΔF508 mutation corrected cells showed the presence of glycosylated and translocated CFTR protein in Western blot analysis. The corrected cells exhibited the functional CFTR channel as demonstrated by YFP assay. Chemically modified *Cas9* mRNA showed reduced or no immunogenicity in *ex vivo* whole blood immune assay.

Conclusion: In conclusion, we have shown that delivery of *Cas9* mRNA together with ssODNs results in functional gene correction of *CFTR* deficient cells.

Suggested Reading:

1. Antony JS *et.al*., 2015. Modified mRNA as a new therapeutic option for pediatric respiratory diseases and hemoglobinopathies. Mol Cell Pediatr 2015, 2(1):11.

2. Firth AL *et.al*., 2015. Functional Gene Correction for Cystic Fibrosis in Lung Epithelial Cells Generated from Patient iPSCs . Cell Reports 12:1385-1390.

Disclosure of Interest: None Declared.

181. Allele-Specific Inactivation of Autosomal-Dominant STAT3 Mutations

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Patients with hyper-IgE syndrome (HIES) due to dominant-negative mutations in *STAT3* suffer from different immunological and non-immunological features. While allogeneic haematopoetic stem cell transplantation is generally not recommended due to potential severe side effects, transplantation of autologous cells in which the mutated *STAT3* allele is specifically inactivated may represent a promising alternative. Here, we have generated allele-specific RNA-guided nucleases (RGNs) to target the most common *STAT3* mutations (H58Y, C328_P330dup, V463del, R382W and V637M). To this end, we have used different Cas proteins and fine-tuned different guide RNA (gRNA) parameters, such as its scaffold, its length and number of mismatched nucleotides between spacer and protospacer. Allele-specificity was initially tested in episomal reporter systems, in which the normal or mutated *STAT3* genes were fused to E2Crimson or EGFP, respectively. The most efficient allele-specific RGNs were then assessed for their ability to discriminate between chromosomal variants of normal and mutant *STAT3* alleles in HEK293T-based reporter cells. For the five mutations tested, allele-specific disruption frequencies ranged from 15% to 30%, without altering the wildtype *STAT3* allele. In conclusion, our results show that allele-specific gene disruption can be efficiently achieved using the RGN system. Ongoing experiments in patient-derived blood cells will underline the potential of this approach to provide a benefit for HIES patients through selective inactivation of the mutated *STAT3* allele.

182. Modulating Transcription Factors Spatial Pattern to Generate Gene-Targeted Therapeutics

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Transcriptional regulation represents the key first functional expression of genetic programs. In the recent years we showed that modulation of transcription via somatic gene transfer of synthetic transcription factors (S-TFs) enables to silence in a potent and specific manner the RHODOPSIN (RHO) gene. Currently, we are exploring a novel strategy to exploit transcription regulation for therapeutic purpose based on the modulation of expression of endogenous TFs, considered for their putative DNA-binding specificity for a DNA sequence by AAV-mediated somatic gene transfer to enable transcriptional

modulation of a target gene and therapeutic effects. Here we show that expression in photoreceptors of an endogenous TF selected for the putative ability to recognize a sequence present on the RHO promoter element, enables robust transcriptional silencing and preservation of retinal functionality in a mouse model of autosomal dominant *retinitis pigmentosa* (adRP) with apparent lack of toxicity.

183. Universal Stem Cell Gene Therapy Platform: Broadening the Genome Editing Arm by Using the Precision Repair Ability of the Non-Homologous End-Joining (NHEJ) Pathway for CRISPR/Cas9-Mediated Blunt-End Integration of Transfecting/Transducing Therapeutic DNA

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Unlike chimeric zinc-finger and TALE nucleases (ZFNs and TALENs), the CRISPR/Cas9 RNA-guided DNA endonuclease generates blunt-ended double-strand breaks (DSBs) in target genomic DNA, thereby promoting the precise DSB repair activity of the non-homologous end-joining (NHEJ) pathway for targeted integration of therapeutic blunt-ended double-stranded DNA (dsDNA). In protocols based on the simultaneous use of a pair of single-guide RNAs (sgRNAs), CRISPR/Cas9 has been shown by others to be very efficient at mediating indel-free excision of genomic DNA (e.g.: paired knock-out) or precise integration of PCR-generated dsDNA (knock-in blunt ligation) in human cells. Such a precision repair ability of the NHEJ pathway for blunt-ended DSBs is in contrast with its well-established error-prone activity that critically hampers gene targeting, i.e. the main genome editing process relying on Homologous Recombination (HR) mediated by the DSB Homology-Directed Repair (HDR) pathway. HDR being restricted to the late S and G2 phases of dividing cells while NHEJ is active both in dividing (G1, S and G2 phases) and quiescent cells, precise NHEJ stands as a complement to basic gene targeting for indel-free genomic integration/substitution of/by therapeutic DNA (e.g.: expression cassettes, regulatory elements, wild-type sequences) through paired sgRNA-mediated CRISPR/Cas9 activity. Such a knock-in blunt reaction involves two DSBs (paired sgRNAs) but does not provide for a desired orientation (no single-stranded homology overhangs); however, it is perfectly fitted to most expression cassettes and to protocols involving a selective or cell sorting step for the transfected/transduced cells/stem cells. Shifting from error-prone to precise NHEJ through CRISPR/Cas9-mediated technology is discussed in light 1) of the four arms of our proposed universal Stem Cell Gene Therapy platform and their synergistic use, 2) of our current protocols designed for CRISPR/Cas9 mRNA nanoparticles aimed at tackling autoimmune diseases, and possibly aging/degenerative disorders and metastatic tumors, and 3) of stem cell engineering strategies in which knock-out of an endogenous gene is mediated by targeted integration of therapeutic blunt-ended dsDNA (e.g.: linear dsDNA; in vivo CRISPR/Cas9-digested minicircles or double-stranded AAV in which relevant sgRNA target sequences have been inserted) comprising a GvHD safety cassette (inducible caspase-9 suicide gene).

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184. Optimizing Safe, Long-Term BTK Expression by Use of Insulator and Endogenous Enhancer Elements in a LV Vector for Treatment of XLA

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X-Linked Agammaglobulinemia (XLA) is a primary immunodeficiency disease in males caused by inheritance of a mutant Bruton's Tyrosine Kinase (*BTK*) allele. BTK is expressed in B cells and myeloid cells and has a key signaling role downstream of the B cell receptor where it is required for both B cell development and activation. As we have previously demonstrated using both gamma-retroviral and lentiviral (LV) vectors, XLA comprises an excellent candidate disease to develop a gene replacement therapy. Restoration of BTK expression confers a selective advantage to developing and mature B cells; thus, correction of a small number of progenitor cells is sufficient to rescue B cell numbers and function in murine models. Similar approaches, if achieved safely, are highly likely to be beneficial in XLA patients. Important considerations for using a randomly integrating gene therapy vector include minimizing the potential for insertional mutagenesis while achieving stable, near endogenous levels of expression in all appropriate cell lineages. In this study, we designed and tested a series of self-inactivating LV vectors with a Ubiquitous Chromatin Opening Element (UCOE) from the *HNRPA2B1-CBX3* locus upstream of a *BTK* promoter (*BTK*p) driving human BTK cDNA expression. We previously showed in a murine XLA model that addition of the 1.5 kb UCOE reduces methylation of the *BTK* promoter, resulting in sustained BTK cDNA expression through serial bone marrow transplants. In addition, a truncation of the UCOE to 0.7 kb maintained its anti-silencing properties, while increasing the vector titer. Here, in an effort to further optimize endogenous-like levels and specificity of transgene expression, we test the utility of predicted *BTK* enhancer elements cloned upstream of the *BTK* promoter in this vector. We identified five intronic enhancer elements distributed across the ~37 kilobase *BTK* locus (each predicted by Genome Segmentations from the Encyclopedia of DNA Elements (ENCODE) Consortium) to comprise evolutionarily conserved, DNase I hypersensitive sites (DHS). These small (150-450bp) DHS elements were cloned in different combinations between the 0.7 kb UCOE and the *BTK*p in our SIN-LV vector (0.7UCOE.*BTK*p.BTK). When used in murine gene therapy experiments, XLA animals treated with LV containing the DHS4 element (0.7UCOE.DHS4.*BTK*p.BTK) exhibited substantial improvement in BTK expression compared to animals treated with the parental LV (0.7UCOE.*BTK*p.BTK). Consistent with these findings, *in vitro* transduction of $CD34^+$ peripheral blood stem cells (collected via G-CSF mobilization and apheresis) from XLA subjects resulted in clinically relevant BTK^+ cells at a viral copy number (VCN) of 1-2 using both vectors. These results suggest that use of bioinformatics to identify potential transcriptional regulatory elements may be beneficial to enhance and/or fine-tune transgene expression in

a gene therapy setting, in both mouse and human CD34 cells. Overall, our data support the consideration of 0.7UCOE.DHS4.*BTK*p.BTK as a candidate clinical vector for LV therapy in XLA.

185. Engineering Therapeutic T-Cells to Recognize and Target Mismatched Alloreactive Human T-Cells

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In solid organ transplants (SOT) or hematopoietic stem cell transplants (HSCT), mismatches in HLA between recipient and donor can lead to rejection of the organ or graft-vs-host disease (GVHD), respectively. Immunosuppressive drugs can mitigate these outcomes, but due to their broadly inhibitory action against immune cells, they increase the risk of opportunistic infections. Alloreactive T-cells that recognize mismatched HLA via their TCR are major mediators of rejection and GVHD. Here we present a strategy to engineer therapeutic T-cells that can eliminate the alloreactive T-cells that contribute to organ rejection and GVHD. We generated a chimeric molecule that fuses beta-2 microglobulin (B2M), a universal component of all HLA class I molecules to the cytolytic domain of CD3 zeta. We hypothesized that this chimeric HLA Accessory Receptor (CHAR) would be able to complex with endogenous HLA class I alpha chains via B2M within the therapeutic T-cell. Subsequently, when an alloreactive T-cell binds a HLA molecule on the therapeutic T-cell it should activate the CHAR that would then mediate elimination of the alloreactive T-cell. To test this hypothesis, we first expressed the CHAR molecule in Daudi cells that lack expression of surface HLA, due to lack of endogenous B2M. We found that expression of the CHAR molecule restored expression of HLA to the surface indicating that the CHAR molecule can complex with endogenous HLA class I molecules. Next we modified virus specific T-cells (VSTs) with the CHAR and found that when CHAR-VSTs encountered T-cells that recognized them as targets, they became activated and degranulated in response. To determine if CHAR-VSTs could eliminate alloreactive T-cells, we cultured them in a mixed lymphocyte reaction (MLR) with allogeneic PBMCs. We found that the outgrowth of activated T-cells from the allogeneic PBMC population was significantly reduced in co-cultures with CHAR-VSTs in comparison to co-cultures with unmodified VSTs. We confirmed the loss of alloreactive T-cells from the PBMCs in a secondary MLR by restimulating them with PBMC autologous to the CHAR-VSTs. Lack of proliferation confirmed that T-cells reactive to the HLA antigens of the CHAR-VSTs had been eliminated. We also confirmed that non-alloreactive T-cells (the majority of T-cells within PBMCs) were retained after exposure to CHAR T-cells, by measuring the frequency and function of virus-specific T-cells within the PBMC population after culturing with CHAR T-cells. We also observed that while CHAR T-cells could eliminate resting alloreactive T-cells within PBMC, they were unable to eliminate pre-activated alloreactive T-cells, since in co-cultures with pre-activated alloreactive T-cells, CHAR-T-cells were eliminated. Therefore, CHAR T-cells as currently designed would require a clinical setting in which alloreactive T-cells would be resting upon first encounter with CHAR T-cells. One example would include living kidney transplantation, where CHAR T-cells derived from the kidney donor could be infused into a recipient prior to transplant to eliminate alloreactive T-cells that could mediate graft rejection. Alternatively in the case of HSCT, recipient CHAR T-cells could be cultured with the stem cell graft prior to infusion to eliminate donor alloreactive T-cells that could attack host tissues. Future experiments will attempt to address elimination of pre-activated alloreactive T-cells that may require further optimization of our CHAR T-cells. In conclusion, we show here a proof of concept that primary human T-cells can be engineered to recognize and target mismatched alloreactive T-cells that can cause severe complications for allogeneic transplant recipients.

186. Lentiviral Gene Therapy for p47^{phox} Deficient Chronic Granulomatous Disease

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Chronic Granulomatous Disease (CGD) is an inherited primary immunodeficiency disorder with an incidence of ~ 1:200,000 live births. This disease is caused by mutations in the NAPDH oxidase, the phagocytic enzyme responsible for pathogen killing. As a result, CGD patients suffer from recurrent bacterial and fungal infections and often life threatening inflammatory complications. Allogeneic Haematopoietic Stem Cell Transplantation (HSCT) remains the only proven curative treatment for patients with CGD. The use of reduced intensity conditioning regimens (to limit toxicity) and the extended use of HLA-matched or single antigen mismatched unrelated grafts can now achieve excellent donor myeloid chimerism and >90% overall survival rate. However, it is not always possible to find a suitable donor and autologous gene therapy has become an attractive alternative option. A phase I/II clinical trial of lentiviral gene therapy is currently underway for X-linked CGD, the most common form of the disease. We propose to use a similar strategy to tackle p47^{phox} deficient CGD, caused by mutations in the *NCF1* gene encoding the cytosolic p47^{phox} subunit of the NADPH oxidase. p47^{phox} deficient CGD is the most common form of autosomal recessive CGD and accounts for approximately 25-30% of patients in the Western world (this is likely to be higher in some consanguineous populations worldwide). We have developed and tested a self-inactivating lentiviral vector containing a codon-optimized p47^{phox} transgene under the transcriptional control of the chimeric cathepsin G/c-fes myeloid promoter (pCCLChim-p47). When used in a p47^{phox} deficient myeloid cell line and in monocyte-derived macrophages from p47^{phox} CGD patients, the lentiviral vector was able to restore p47^{phox} expression and oxidase activity to normal levels. In a murine model of stem cell gene therapy for p47^{phox} deficient CGD, the pCCLChim-p47 vector induced high expression of the

p47^{phox} protein in granulocytes from blood and bone marrow of gene therapy treated mice and restored levels of NADPH-oxidase activity that were comparable to those found in wt animals (with an average of~ 1 vector copy per cell). As expected, the expression of the p47^{phox} protein was mainly confined to myeloid cells in blood, bone marrow and spleen. The percentage of functional neutrophils remained stable over time up to six months, suggesting that the vector is not prone to epigenetic inactivation. The presence of corrected granulocytes in secondary transplanted animals also indicates that we can successfully transduce haematopoietic stem cells. Overall this study shows that the pCCLChim-p47 vector is a promising tool for the clinical gene therapy of p47^{phox} deficient CGD.

187. Prevalence of Neutralizing Antibodies Targeting Two Novel Clade F AAV in Human Sera

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A novel group of adeno-associated viruses have been cloned from human CD34+ hematopoietic stem cells (AAVHSC). Capsid sequence analysis has shown that these vectors map to AAV Clade F of which AAV9 is the prototypic member. Since Clade F viruses are emerging as key vectors for gene therapy, it is imperative to understand the factors that regulate their pharmacokinetics and bio-distribution in the intact animal. One such factor is the presence of neutralizing antibodies (Nab) within the blood that may opsonize the vectors and block AAV-mediated cellular transduction. Depending on the dose of AAV administered, even low titers of Nab can reduce AAV-mediated gene delivery. In the present study, the human prevalence and titers of Nab that block the transduction of cells by AAVHSC15 and AAVHSC17 were assessed in a representative human population and compared to those of AAV9. Nab levels were measured in a set of 100 unique mixed-race (34:33:33, Black:Caucasian:Hispanic) and sex (49% female, 51% male) human sera collected within the United States. All sera were heat-inactivated by incubation prior to assay. Two cellular assays at two separate laboratories were used to measure Nab: 56 unique sera were tested in HuH7 cells and 44 unique sera were tested in 2V6.11 cells with vectors packaging either a chicken beta actin (CBA)-promotered *LacZ* or a Firefly Luciferase transgene, respectively. A ten-sample overlap of sera was also included to test concordance between the two laboratories. Nab prevalence was assessed using 1/16-1/64 dilutions of each serum sample whereas Nab titers of positive samples were assessed using a two-fold dilution series (1/5 to 1/1280) of each sera. Concordance of Nab levels measured in the two cell assays was 100%. For AAVHSC15, AAVHSC17, and AAV9, 24/100 (24%) , 21/100 (21%), and 17/100 (17%), respectively, of all sera tested were seropositive for Nab. Over a wide range of dilutions, each Nab positive sera (24 in total) blocked, by cross-reactivity, the transduction of each AAV with titers for 50% neutralization falling equally into four groups: less than or equal to 1/25, 1/50, and 1/100, and greater than or equal to 1/150 for all three vectors. In the latter group, which represented 6% of Nab positive sera, Nab titers of 1/150 (lowest) to 1/340 (highest) were observed indicating that the majority of Nab positive sera were of low titer. These data demonstrate that approximately 80% of sera in the human population tested were seronegative for Nab to Clade F AAV, enabling the use of AAVHSC as therapeutic vectors for human diseases.

188. Safety & Efficacy of Fetal Gene Therapy: A Non-Human Primate Model

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Abstract Body: Fetal gene therapy (FGT) is sometimes the only option available to treat genetically damaged fetuses, in cases where they are not likely to survive till term. FGT can also be useful in arresting disease pathology, to allow for further intervention once the fetus has been delivered. Additionally, fetuses are thought to be relatively pre-immune, minimising the risk of adverse reactions. We have thus examined the safety and efficacy of FGT in a non-human primate (NHP) model, up to 90 months of age. In our study, NHP fetuses were injected with scAAV-LP1-hFIX (AAV5 or AAV8 pseudotypes) in early (0.4G, n=6) or late (0.9G, n=5) gestation. Fetuses given scAAV-LP1-hFX were injected at 0.4G (n=6). Early FGT (eFGT) recipients were given 1-10 x 10^{10} vg/fetus while late FGT (lFGT) recipients were given 4 x 10^{12}vg/fetus. Offspring were serially monitored for transgene and immune expression, and 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 if there was hepatotoxicity. Liver transaminase levels were also serially monitored. Liver DNA was analysed for AAV integration through LAM-PCR and next-generation sequencing. Transgene expression of 1% of physiological levels (0.05mg/ml for hFIX, 0.1mg/ml for hFX) were considered therapeutic - offspring exhibiting sub-therapeutic expression (n=5) were given post-natal vector infusions, and examined for T-cell activation and neutralising antibody (NAb) responses. Generally, higher transgene levels were exhibited in males. AAV8 recipients also showed higher transgene levels as compared to AAV5 recipients (lFGT-hFIX: median expression of 19% vs 8%; eFGT-hFIX: 15% vs 2%; eFGT-hFX: 4.4% vs 0.6%). lFGT recipients showed higher transgene expression (AAV5: median expression of 12% vs 2%; AAV8: 47% vs 16%). Of note, hepatic vector copy numbers (VCN) followed the same trend, hinting that stability of transgene expression could be due to vector integration. Integration analyses indicate random, non-repeating, low-level integration. Livers showed no abnormality, neither visually nor histologically, and transaminase levels stayed largely within range. Anti-transgene immune response was negligible in all recipients, while lFGT recipients showed milder anti-AAV response even though transgene levels were higher. Recipients of post-natal boosts exhibited NAb response, but showed little T-cell activation. No significant change in humoral immune response and transgene expression was observed. Our results show that a single dose of AAV in utero, regardless of gestational age, is able to effect stable therapeutic expression in FGT recipients up to 90 months. Instead of vector dosage, vector pseudotype and gender of FGT subject are more likely to determine if therapeutic transgene expression is achievable.

Although post-natal boosts indicated the presence of NAb, the lack of a humoral immune response hints at central tolerance to AAV. Overall, safety and efficacy data accumulated so far have been encouraging.

189. Gene Editing in Fanconi Anemia: CRISPR/Cas9-Mediated Correction of *FANCD1* Gene in Patient Primary Cells

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The treatment for the bone marrow failure syndrome Fanconi anemia is hematopoietic cell transplant that can be associated with significant side effects and safer therapies are needed. Gene editing, utilizing programmable nucleases, holds great potential to change the treatment paradigm for Fanconi anemia by correcting the underlying genetic causes. This has been accomplished so far in transformed patient-derived cells or induced pluripotent stem cells. The correction of Fanconi anemia patient primary fibroblasts; however, has not yet been achieved without incorporation of selectable markers. Here we aimed to address this question and correct the mutation in *FANCD1* primary patient fibroblasts employing the clustered regularly interspaced short palindromic repeats/Cas9 system. We generated a candidate effectively targeting the *FANCD1* 886delGT mutation (PAM site located 4 bp downstream of the mutation). The nuclease and the corrective donor carrying the wild-type *FANCD1* sequence were electroporated into the patient-derived fibroblasts as plasmid DNA and a 121 bp-long ssDNA oligonucleotide, respectively. Given the severely reduced level of homologous recombination in *FANCD1*-deficient cells, gene editing of *FANCD1*mut cells represents a substantial technological challenge. Nonetheless, using inhibitors of poly ADP-ribose polymerase as a selective marker we isolated six monoclonal populations of patient-derived gene-corrected fibroblasts (out of 30 generated clones). In 6/6, we confirmed by molecular analysis the corrected genotype as well as proper splicing of the edited gene/cDNA. Moreover, we also showed a restoration of FANCD1 protein function in the gene-corrected cells as demonstrated by efficient FANCD1-dependent trafficking of RAD51 recombinase to the nucleus after exposure to DNA damaging agents. Altogether we showed our ability to correct mutations in *FANCD1* gene in primary patient cells. Our results represent a significant step forward for gene editing-based therapies in support of translational application. Funded by GACR 17-04941Y, Tulloch chair and patient support organizations.

190. Addition of dmPGE2 Enhances BM and mPB CD34+ Cell Transduction Preserving *In Vivo* Reconstitution Potential, Differentiation and Integration Profile of Globin-LV

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Successful gene therapy of beta-thalassemia (Bthal) by gene addition in hematopoietic stem cells (HSCs) is dependent on the achievement of therapeutic level of hemoglobin produced by corrected erythroid cells. The broad spectrum of mutations and severity in Bthal, along with an expected variability on gene expression/LV copy dependent on integration site (IS), require a continuous effort in the development of more efficacious approaches of gene therapy. Towards this objective, we aimed to enhance the therapeutic potential of GLOBE lentiviral vector (LV) by acting on transduction methods, increasing transduction efficiency along with VCN/cell. We explored and compared different transduction protocols on relevant HSC sources for gene therapy, i.e. $CD34^+$ derived from bone marrow (BM) and peripheral blood mobilized by Plerixafor with or without G-CSF (MPB). We shortened culture time in respect to current protocol and we introduced the use of $dmPGE_2$, recently employed in clinics to increase engraftment in patients transplanted with cord blood. Our results showed that the addition of $dmPGE_2$ increases *in vitro* VCN and transduction efficiency, depending on the HSC source (BM *vs* MPB). The effect of this molecule on $CD34^+$ cells is depending on its receptors expression. Indeed, we found differential EP2 expression on the different primitive hematopoietic subsets. IS analysis in cells transduced in presence or absence of PGE_2 showed no differences in IS distribution, in top target genes and gene ontology categories among the protocols and cell sources. Following transplantation of transduced cells in NSG mice, the presence of $dmPGE_2$ during transduction did not affect the graft composition and high transduction efficiency was maintained into long-term repopulating cells. Ongoing molecular and functional studies will unravel mechanisms of $dmPGE_2$ action on $CD34^+$ cells and its impact on the biological and functional activities of transduced cells.

191. Dissection of Viral Reservoir and Persistence of HIV-1 In Vivo

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The persistent infection with the human immunodeficiency virus type 1 (HIV-1) represents a global health problem. The virus integrates into the genome of host cells and replicates. Finally, HIV-1 infection leads to the acquired immunodeficiency syndrome (AIDS). Although combined antiretroviral therapy can suppress the viral replication, it is not possible to eradicate the latent viral reservoir completely. Integrated viruses from this reservoir evade therapy and might be re-activated in the patient. It has been shown that integration sites on certain gene loci are able to promote the persistence of HIV-1 and the expansion of infected cell clones, even during combined antiretroviral therapy. The aim of the study was to dissect the reservoir and persistence of infected cells over time, before therapy (1-7 time points) and after therapy (1-4 time points). Therefore, we aimed to identify and characterize the respective HIV-1 integration loci by using the standard LAM-PCR (linear-amplification mediated PCR). Additionally, we established Target Enrichment Sequencing (TES) as method for HIV-1 integration site analysis and to gain insights about virus coverage and possible mutations.

By applying LAM-PCR, we were able to detect a total number of 2,096 HIV-1 integration sites in 32 HIV patient samples from peripheral blood. Around the half of those integration sites were located within gene regions. Clonal persistence over time was observed in nearly all patients, but mostly before therapy start. Regarding possible hotspots for HIV-1 integration, several genes could be identified as hotspots, which harbored more than one integration site locus. Among else, six integration loci were detected in RUNX1, TMEM132C and EHMT1. In contrast, HIV-1 integration hotspots described in previous studies from other groups, e.g. BACH2, MKL2 or STAT5B, could not be confirmed as hotspots in this study.

Of interest will be the ongoing comparison of LAM-PCR with Target Enrichment Sequencing in our patient samples. Besides identification of integration sites, TES allows us to align sequencing data to the HIV-1 reference genome to gain information about virus quantification and mutations in the viral genome in patient samples over time. Results for the first set of probes are available for both in vivo and in vitro samples. In case of a second set of probes, results for in vitro samples already show a 1-log improvement regarding the on-target efficiency compared to the first set of probes. Further optimization is ongoing for in vitro and in vivo samples.

Comparing the results with other studies, persistence of HIV-1 integration sites and thus HIV-1 infected cells over time can be confirmed. Possible hotspots found in this study differ from those of other studies, which might be attributed to our study focus of analyzing samples before and after start of combined antiretroviral therapy. The study of further patient samples will give more insights.

192. Targeted Gene Correction for the Treatment of Wiskott-Aldrich Syndrome

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Wiskott-Aldrich syndrome (WAS) is an X-linked primary immunodeficiency characterized by severe platelet defects, defective cellular and humoral immunity, recurrent infections and development of autoimmune diseases and cancer. This disease is caused by mutations in the *WAS* gene which encodes for the WAS protein (WASp), required for the regulation of the actin cytoskeleton in multiple hematopoietic cell lineages, for proper platelet production and lymphoid cell function. Currently, allogeneic stem cell transplantation constitutes the only available cure for WAS. Gene therapy approaches using lentiviral vectors aiming to restore WASp expression in hematopoietic stem cells (HSPCs) of WAS patients showed encouraging results, with good immune reconstitution in most patients. Although effective, viral vectors carry a potential risk of genotoxicity and non-physiological transgene expression in target cells and 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 directly knock-in a wild-type *WAS* cDNA close to its endogenous start codon, allowing transcriptional regulation from *WAS* endogenous promoter and the functional correction of the mutations in the *WAS* gene in primary human 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 levels of gene editing, with indels rate of up to 80% as assessed by T7 endonuclease and TIDE assays. Disruption of the *WAS* locus was further confirmed by the absence of protein expression and the impaired cytoskeleton organization in edited single-cell clones. The best performing Cas9/gRNA complex was further tested in human HSPCs from different donors, reaching up to 50% of editing efficiency. We next created a synthetic AAV6 donor template for homology-directed repair (HDR) that contains a PGK promoter-driven GFP cDNA flanked by ~800bp *WAS* homology arms and an homology-independent targeted integration (HITI) AAV6 donor template containing the same PGK-GFP reporter cassette flanked by the gRNA target site. We will compare the efficiency of targeted gene addition achieved with both platforms by delivering the AAV6 donor templates to hematopoietic cell lines and HSPCs along with the Cas9/gRNA ribonucleoprotein complex, in order to determine the best editing strategy that support a viable therapeutic approach for the treatment of WAS.

193. Developing Alternative Conditioning Regimens Using Lamprey-Antibody Based Immunotoxins

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Transplantation of genetically-modified stem cells is becoming a new paradigm for treatment of rare genetic disorders. Adenosine deaminase deficiency (ADA-SCID) is the first monogenic immune disease thought to be curable by an approved gene therapy product shown to be efficacious in pediatric patients. This treatment is a major milestone for hematopoietic stem cell transplantation (HSCT) gene therapy not only because of its curative potential but also because it reduces the risks of the current standard of care. HSCT gene therapy involves the transplantation of autologous cells eliminating the risks of graft versus host disease or graft rejection. However, this method of gene transfer requires the depletion of bone marrow cells prior to transplant in order to facilitate engraftment of genetically-modified cells. Chemotherapeutic alkylating agents are profoundly bone marrow ablative and used clinically as a preparative regimen for stem cell transplantation. However, these drugs are also associated with toxicities including hepatic veno-occlusive disease, thrombocytopenia, secondary malignancies, and sterility. Developing non-genotoxic drugs that can effectively ablate hematopoietic stem cells (HSCs) is among the most important obstacles to overcome as the field pushes to expand this therapy to other less severe monogenic diseases. Our approach has been to identify new HSC epitopes or cell surface targets using the lamprey adaptive immune system, a primitive jawless vertebrate that diverged from our common ancestor 550 million years ago. Lampreys secrete variable lymphocyte receptors (VLRs), similar in function, but not homologous, to IgG, which mediate antigen recognition and aid in anticipatory immune responses. In contrast to IgGs, VLRs are naturally single chain proteins that exhibit a high degree of antigen specificity. Additionally, recombinant VLRs are being expanded into multiple avenues of research including CAR T-cell therapies and cancer diagnostics. We have developed a unique platform in which our laboratory can generate antigen-specific VLRs from lampreys immunized with primary murine bone marrow derived $Kit^+Sca\text{-}1^+Lin^-$ (KSL) hematopoietic stem and progenitor cells. Lampreys demonstrated a polyclonal immune response resulting in the identification of 11 unique VLR sequences specific to Lin^-, but not Lin^+ cells. These sequences were sub-cloned into expression plasmids facilitating the purification of recombinant VLR proteins fused to the Fc region of murine IgG. These fusion proteins were directly conjugated to fluorophores and demonstrated to display discrete binding to primary murine KSLs detectable by flow cytometry. Hydrodynamic injection of VLR-Fc expression plasmid resulted in detectable plasma levels of VLR antigen measurable by ELISA. Given this data, we anticipate VLR-Fc fusion proteins will serve as an excellent binding domain for anti-HSC immunotoxin development. We are currently examining the capacity of our VLR-toxin conjugate to deplete murine hematopoietic stem cells in vivo.

194. Competitive Transplants in Rag1$^{-/-}$ Mice Support the Feasibility of Genome Editing for the Treatment of RAG1 Defect

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Recombination activating gene 1 (RAG1) is a molecule tightly regulated in lymphocyte differentiation and its inappropriate expression leads to chromosomal translocation or development of autoimmune reactions. Mutations in *RAG1* gene cause Severe Combined Immunodeficiency (SCID), characterized by the lack of circulating lymphocytes and can also result in a broad spectrum of clinical phenotypes with immune dysregulation and autoimmunity. RAG deficiency is fatal unless treated by Hematopoietic Stem Cell Transplantation (HSCT), although data obtained from retrospective studies indicated partially satisfactory results when HSCT is performed with a related HLA-partially compatible donor. Gene therapy has been proposed as an alternative treatment, however preclinical studies in *Rag1*$^{-/-}$ mice highlight the need of a physiological control of gene expression in order to cure the disease and minimize potential risks of cancer and autoimmunity. Genome editing (GE) strategy represents a feasible and valid method to cure genetic defects caused by mutations in genes tightly regulated at the first phases of lymphocyte differentiation. Because of the reduced number of corrected $CD34^+$ cells expected by available GE protocols, we have evaluated whether a small number of corrected HSC can correct the immunodeficiency. We performed competitive transplantation in lethally or sublethally irradiated *Rag1*-/- mice receiving 5% or 10% wild-type (wt) lineage negative (Lin^-) cells mixed with *Rag1*$^{-/-}$ Lin^- cells. As positive and negative controls, *Rag1*$^{-/-}$ mice received 100% wt (BMT-WT) or 100% *Rag1*-/- (BMT-KO) Lin^- cells, respectively. Kinetics of immune reconstitution and chimerism were evaluated over time by flow cytometry and at sacrifice 5 months after transplant. We found that 10-5% wt cells in *Rag1*-/- mice were sufficient to partially overcome T and B cell differentiation blocks in thymus and bone marrow, respectively. This results in a progressive and stable immune cell reconstitution in the periphery, although lymphocyte counts were still lower in *Rag1*-/- mice with low doses of wt cell as compared to BMT-WT mice. However, peripheral T cell compartment showed a normal CD4/CD8 ratio, proportions of naïve and memory/effector cells and normal *in vitro* function. B cell reconstitution and function normalization of BAFF levels and the production of all immunogloblulin classes and specific antibodies to T-cell dependent antigens were observed. In conclusion, we demonstrate that minimal doses of corrected cells are able to reconstitute immune system in *Rag1*-/- mice, although T and B cell counts remain low. This finding supports the feasibility of GE strategy in RAG1 deficiency, and indicates the need to develop novel conditioning regimens more effective in stem cell niche depletion

195. Resistance of Mouse Hematopoietic Stem and Progenitor Cells to the Genome-Editing with Cas9

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Gene therapy in hematopoietic stem cells has been successfully conducted to treat genetic disorders such as severe combined immunodeficiency (SCID). However, viral vectors used in gene therapy may cause random transgene integration and might result in dysregulated gene expression. Thereby, targeted repair of mutations causing disease is highly desired. The targeted repair is to replace the mutation with a correct sequence in site-specific manner by homologous recombination (HR). Recently, gene-editing tools have been developed to induce double strand breaks (DSBs) that enhance HR. Among them, the clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9) system is most convenient and robust system. We used *Staphylococcus aureus* Cas9 (SaCas9, 1053 amino acids) that is smaller than widely-used *Streptococcus pneumoniae* Cas9 (SpCas9, 1368 amino acids) with expectation of easier delivery to somatic cells. In this study, we aimed to edit genome of hematopoietic stem and progenitor cells (HSPCs) by using CRISPR/Cas9 in clinically relevant setting. To this end, we generated X-linked SCID mice to mimic the human SCID mutation, and delivered SaCas9 to edit the interleukin-2 receptor common gamma chain (IL2Rg) gene in HSPCs, which would provide future prospects of targeted repair for SCID patients. We generated SCID mice with point mutations in the mouse IL2Rg gene by micro-injection of SpCas9 mRNA and synthesized single guide RNA (sgRNA), targeting the exons 2, 3 and 4 of mouse IL2Rg, to mouse fertilized oocytes. We obtained total 25 out of 35 founder lines with mutations and SCID phenotypes. To deliver SaCas9 to HSPCs, we constructed human immunodeficiency virus-based lentiviral vector that simultaneously expresses SaCas9, EGFP and sgRNA. The sgRNA was designed to introduce a DSB with Cas9 in IL2Rg gene. We tested whether the SaCas9-expressing lentiviral vector cause the DSB in NIH3T3 cells. Transduction efficiency was examined by GFP expression with flow cytometry and we performed T7 endonuclease Surveyor assay to detect non-homologous end joining (NHEJ) after the DSB. The SaCas9-expressing vector efficiently transduced NIH3T3 cells (30-90%). We detected the NHEJ in the IL2Rg locus. Then, we examined whether the same lentiviral vector could induce the DSB in mouse HSPCs (Lin- c-Kit+ bone marrow cells). Up to 37% (8-37%, n = 11) of HSPCs was transduced, which levels are enough to detect the NHEJ by the Surveyor assay. However, the NHEJ has never been detected in the HSPCs. The samples have now been subjected to the deep sequencing to examine if a low frequency NHEJ was introduced to the target site. In summary, we demonstrated successful genome editing by SpCas9 and SaCas9 in mouse fertilized oocytes and NIH3T3 cells, respectively, although NHEJ following DSB was undetectable in HSPCs. These observations suggest that HSPCs are possibly resistant to Cas9. Elucidating the mechanisms of resistance of HSPCs to Cas9 will be a key step for realizing the targeted repair in HSPCs.

Immunological Aspects of Gene Therapy and Vaccines I

196. Second Generation Anti-Cocaine Vaccine Based on Modified Adenovirus Capsid Proteins

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Cocaine addiction remains a significant public health issue, accounting for approximately 40% of drug related emergency department visits and 7,000 deaths annually. Currently, the only treatment option for cocaine addicts is behavioral intervention. Toward the goal of developing a vaccine that would prevent cocaine from reaching its cognate receptors in the brain, we are currently testing in the clinic a 1st generation anti-cocaine vaccine (dAd5GNE) consisting of GNE, a hapten analogous to cocaine, coupled to the highly immunogenic proteins of a disrupted serotype 5 $E1^{-}E3^{-}$ adenovirus (Ad). In experimental animals, this vaccine generates high-titer anti-cocaine antibodies that successfully sequesters cocaine in the blood, preventing the drug-induced reward. Looking forward to the development of a 2nd generation vaccine, we hypothesized that we could enhance the potency of the current anti-cocaine vaccine by incorporating immunomodulating sequences into the hyper-variable regions (HVR) 1, 2, and 5 of hexon, the capsid protein largely responsible for the immunogenicity of Ad. The hexon modifications were derived from several categories of immunomodulating strategies, including: (1) the promiscuous T-helper epitope from tetanus toxin ($TT_{830-844}$); (2) the adjuvanting sequence from the invasin protein of *Yersinia pestis*; and (3) the addition of 5-lysine residues, to increase the number of conjugation sites for GNE attachment. Each of these modified adenovirus constructs were disrupted and conjugated to GNE in an identical fashion as dAd5GNE and evaluated compared to current dAd5GNE vaccine in BALB/c mice at (doses of 1.2 μg and 4 μg, administered intramuscularly) Using a 0, 4, 8 wk prime-boost regimen, the titers after the 3rd vaccination at the 4 μg dose were $1.4x10^5$, $7.0x10^5$ and $1.4x10^6$ for the T-helper, invasin and additional lysine constructs, respectively, with the first two not significantly different from the dAd5GNE vaccine (titer $p>0.06$, for both). In contrast the lysine modified hexon vaccine resulted in a significantly more potent vaccine ($p<0.04$). Consistent with our previous data demonstrating that higher titers mediate greater efficacy, mice vaccinated with the disrupted adenovirus containing the lysine modified hexon had a 3.6-fold increase in the ratio of cocaine sequestered in the blood to cocaine in the brain following intravenously administered cocaine as compared to the first generation dAd5GNE. The increase of cocaine sequestered in the serum and the reduction of cocaine crossing the blood brain barrier were significantly different than that observed in the dAd5GNE vaccinated mice ($p<0.04$ for serum and $p<0.009$ for the brain). In summary, addition of lysines to the Ad5 hexon hypervariable region enhances the potency either by increasing the number of conjugated GNE moieties or through increasing the adjuvant effect of the 1st generation dAD5GNE anti-cocaine vaccine, a strategy that could be developed as a 2nd generation vaccine.

197. Comprehensive Analysis of Innate and Adaptive Immune Response to AAV2 Capsid in Healthy Donors

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Adeno-associated virus (AAV) vector occupies the front scene of gene therapy since it gained merit in numerous proof-of-concept and safety studies in animal models. However by now, several clinical trials showed that this vector, though originating from a non-pathogenic virus, is not ignored by human immune system. Indeed, in some subjects undergoing gene therapy, loss of transgene expression was seen and was accompanied by simultaneous increase of AAV-specific $CD8^+$ T cells in peripheral blood. For this reason, there is an urgent need for a deeper understanding of immune response directed against AAV capsid.

Here, we present a comprehensive analysis based on *ex vivo* experiments using PBMCs obtained from healthy donors. First, using mass cytometry (CyTOF) we have concomitantly characterized cytokine secretion, activation and exhaustion markers in 11 cellular subsets in response to AAV2 capsid. In three out of four tested donors, AAV capsid triggered secretion of TNFα and IFNγ as well as overexpression of HLA-DR in the $CD16^{bright}CD56^{dim}$ NK cells. These results were confirmed on higher number of donors with intracellular cytokine staining and flow cytometry or Luminex technology, which measures cytokines released into culture medium. Further analysis showed that AAV-responsive NK cells form a small (0.4% ±0.1) but well defined population with a distinct KIR repertoire. To our knowledge, this is the first time that activation of NK cells in response to AAV is reported. Although these cells do not seem to be cytotoxic, thanks to the secreted cytokines, they may play a role in dendritic cell (DC) maturation and thus ameliorate presentation of AAV epitopes. Indeed, in the presence of AAV, we could identify a DC subset secreting Il-1β and Il-6 pro-inflammatory cytokines, also known to induce B cell differentiation into antibody-producing plasma cells. As expected, stimulation of PBMCs with AAV, without the need for exogenous cytokines, resulted in increased plasmablast number in cell cultures. A degree of this increase was strictly correlated with the anti-AAV antibody titers in plasma of donors. Since further experiments using blocking anti-Il-1β and anti-Il-6 antibodies showed that AAV-induced B cell differentiation requires both cytokines, we are currently further investigating this phenomenon.

Regarding adaptive immune response, CyTOF analysis showed that in majority of tested donors memory $CD8^+$ T cells responded by TNFα but not IFNγ secretion, while $CD4^+$ T cells did not seem to be responsive. Classical flow cytometry analysis in numerous donors, including staining of AAV2-TMr^+ cells, confirmed that AAV2-specific $CD8^+$ T cells lacked IFNγ secretion and were unable to upregulate CD25 when restimulated. Such characteristics can suggest "helpless" phenotype and explain overall weak immune response to AAV2 compared to other viral capsids.

Future experiments will further explore the complex interactions between multiple immune players activated by AAV and identified in this study.

198. Circulating Anti-AAV5 Neutralizing Antibody Titers up to 1:1031 Do Not Affect Liver Transduction Efficacy of AAV5 Vectors in Non-Human Primates

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Ongoing clinical trials using adeno-associated virus (AAV) vectors for therapeutic gene delivery targeting the liver have shown promising results. However, there is a general concern that systemic delivery, and hence efficacy, of AAV vectors may be negatively influenced by the presence of circulating pre-existing anti-AAV neutralizing antibodies (NAB). Currently, patients who present levels of anti-AAV antibodies considered as low are excluded from gene therapy trials. Indeed, low levels of pre-existing anti-AAV NAB have been reported to impair liver transduction by AAV serotype 8 (reported titers of 1:5) in non-human primates (NHPs) (Jiang et al., Blood 2006) and by AAV serotype 2 (reported titers from 1:3.3 and 1:10) in an *in vivo* mouse model of passive immunity (Scallan et al., Blood, 2006).

Considering that NAB titers are relevant for the initial transgene expression, we sought to assess the impact of circulating anti-AAV5 NAB levels on the liver transduction efficacy of an AAV5-based vector delivered systematically. The sera of 14 NHPs were assessed for the levels of pre-existing anti-AAV5 NAB before intravenous administration of an AAV5 vector (AAV5-hFIX) at a dose of 5e11 gc/kg (n=3), 5e12 gc/kg (n=5), 2.5e13 gc/kg (n=3) or 9.3e13 gc/kg (n=3). Anti-AAV5 NAB titers were assessed using a specific bioassay sensitive enough to detect NAB at titers as low as 1:1. Transduction efficiency was assessed by measuring transgene proteins levels in plasma 7 days after vector infusion, and vector DNA in the liver 6 months after vector infusion (post mortem).

All animals displayed pre-existing anti-AAV5 NAB, at titers ranging from 1:57 to 1:1031. Within each dose group, successful and comparable transduction was achieved, independently of the level of pre-existing anti-AAV5 NAB. At sacrifice, the amounts of AAV5 vector DNA and transgene mRNA in the liver were directly proportional to the injected dose of AAV5 and found similar within animal groups tested regardless of the level of anti-AAV5 NAB at pre-administration.

In summary, our data demonstrate that anti-AAV5 NAB titers up to at least 1:1031 do not impact transduction of the liver by AAV5 in NHPs.

199. Delivery of Therapeutic Monoclonal Antibody Genes for Prophylaxis of Respiratory Syncytial Virus Infection

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Respiratory syncytial virus (RSV) infection is the single most common cause of hospitalisations in infants under 1 year of age. Globally, the virus is responsible for an estimated 33.8 million incidents of acute lower respiratory infections in children younger than 5 years (Lancet 375:1545, 2010). In the US alone, approximately 2.1 million children under 5 years of age require medical attention every year due to RSV infection, of which 3% need hospitalisation (N Engl J Med 360:588,

2009). There is currently no vaccine against RSV. Prophylaxis with monoclonal antibody palivizumab (Synagis, MedImmune) is effective, and reduces hospitalisation rates by 55% in infants up to 6 months of age (Pediatrics 102:531, 1998). Next generation antibodies are being developed, with improved efficacy and half-life. The use of antibodies for RSV prophylaxis is however costly due to the complexity of large-scale manufacture, and thus only offered to select vulnerable infant populations, such as children with chronic lung disease, congenital heart disease, or preterm infants. Therefore we are developing gene delivery approaches to establish 'protein factories' for direct expression of palivizumab and other antibodies in patients. Recombinant adeno-associated virus (rAAV2/8) was used for delivery to the gastrocnemius muscle of BALB/c mice, with transgene expression under the control of the CASI promoter (Nature 481:81, 2011). Recombinant Simian Immunodeficiency Virus (SIV) pseudotyped with Sendai virus envelope proteins F and HN (Thorax 72:137, 2017) (rSIV.F/HN), with transgene expression from the hCEF promoter (Nat Biotech 26:549, 2008) was delivered to the mouse lung via insufflation. We performed proof of concept studies in mice using vectors expressing secreted *Gaussia* luciferase (GLux) reporter protein. Delivery of either vector (1E6, 1E7 or 1E8 TTU of rSIV.F/HN delivered intranasally in 100 μl; 1E9, 1E10 or 1E11 GC of rAAV2/8 delivered IM in 40 μl) resulted in sustained reporter expression in the lung lumen, measured in broncho-alveolar lavage fluid (BALF) ($p<0.001$ and <0.01 for lentivirus and AAV, respectively) for 12 months. Both vectors also resulted in systemic reporter (GLux) expression in the circulation ($p<0.001$ for both vectors) for at least 1 year. rAAV2/8 delivered intramuscularly achieved >800-fold greater GLux expression in the circulation compared with the lentiviral vector, but intranasal delivery of rSIV.F/HN yielded >900-fold greater reporter levels in the BALF compared with AAV. Next, recombinant SIV and AAV vectors were constructed expressing palivizumab. Following delivery to mice, therapeutically relevant serum levels of the antibody were achieved using rAAV for at least 6 months (89.3 μg/ml at 6 months post-delivery of 1E11 GC/mouse; $p<0.001$). Furthermore, both delivery vectors resulted in palivizumab secretion into the lung at 1 month ($p<0.05$ and 0.001, respectively), with very high levels of antibody (313 ng/ml; $p<0.001$) detected in BALF at 6 months with rAAV. This was in contrast to the trend previously observed with GLux, where rSIV.F/HN was superior for delivery to the lung lumen. We are currently undertaking studies to determine whether observed levels of palivizumab are protective against RSV infection in animal models. We speculate that, if successful, this approach could significantly reduce per dose treatment costs, which could allow for wider use of prophylaxis against RSV infection in the continued absence of an effective vaccine.

200. Assessment of Immune Responses to scAAV9-HEXM in Tay-Sachs Mice

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Tay-Sachs disease (TSD) and Sandhoff disease (SD) are neurodegenerative diseases caused by the accumulation of GM2 gangliosides (GM2) resulting from deficiency of the heterodimeric isoenzyme β-Hexosaminidase A (HexA). TSD is due to mutations in the α-subunit (*HEXA*) of HexA and SD results from mutations in β-subunit (*HEXB*) of HexA. Gene therapy approaches to treat these diseases have been hampered because of the hetero-dimeric nature of the enzyme required. A new variant of the human α-subunit of HexA, containing the critical sequences from β-subunit that can form a stable homodimer (HexM), had been designed and shown to be capable of hydrolyzing GM2 independent of the endogenous subunit (Molecular Therapy, 2016). *HEXM* has been proven to be therapeutic for both TSD and SD (Human Gene Therapy, 2016). Since this functional homodimer gene can be packaged within a single self-complementary AAV genome, it has significant advantages for treating both TSD and SD using approaches for widespread CNS gene transfer. However, while an anti-HexA immune response might be expected in TSD patients or mice that do not express any HexA protein, the immunological profile of the engineered HexM protein in HexA knockout (TS) or WT mice was not known. A study was designed to assess the immune response to AAV gene transfer in TS mice using scAAV9-h*HEXM*. The study included analysis at 3 weeks and 6 weeks after intravenous injection, in males and females (n=24 each), and in heterozygous (het) versus TS mice (n=24 each). The results showed that the delivery of scAAV9-h*HEXM* induced anti-HexM T cells in both Het and TS mice when compared to vehicle injected mice. An ELISA was performed to quantify the level of anti-HexM antibodies in sera. While mice treated with vehicle did not generate anti-HexM antibodies, all mice treated with scAAV9-h*HEXM* developed an anti-HexM antibody response ($p<0.000001$), which more than doubled upon secondary immunization with HexM protein. There was no significant difference in antibody levels between Het and TS mice. The biodistribution of scAAV9-h*HEXM* was determined in liver and brain samples of mice from the study. Across all different treatment combinations, there was a reduction in the copy number from 3 weeks to 6 weeks post-injection in the liver ($p=0.028$ in TS mice, 0.055 for Het), which could indicate a cytotoxic lymphocyte-mediated clearance of transduced cells. While there was a trend towards a reduction in the brain, it was not significant. Notably, in spite of this immune response, scAAV9-h*HEXM* has already been shown to improve the overall survival and function of SD mice (Human Gene Therapy, 2016). We are currently evaluating immunosuppression strategies to make scAAV9-h*HEXM* gene therapy safer, which we predict could also improve its efficacy in treating both TSD and SD.

201. LV.InsulinB9-23/Anti-CD3 mAb Inhibits Recurrence of Autoimmunity in Diabetic NOD Mice After Islet Transplant

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Type 1 diabetes (T1D) is an autoimmune disease resulting in complete destruction of insulin-producing beta pancreatic cells by auto-reactive T cells targeting islet-associated antigen(Ag)s. Induction of Ag-specific tolerance represents a potential therapeutic option for T1D. We previously showed that systemic administration of a single dose of LV.ET.InsB9-23.142T (LV.InsB), enabling stable expression of InsB9-23 in hepatocytes arrests beta-cell destruction in NOD mice, the spontaneous murine model of T1D, at advanced pre-diabetic stage maintaining stable normoglycemia by generating InsB9-23-specific FoxP3+ T regulatory cells (Tregs). Moreover, LV.InsB in combination with a suboptimal dose (1X 5μg) of anti-CD3 mAb reverts overt diabetes halting preserving residual beta-cell mass.

In the present study we tested the efficacy the LV.InsB/anti-CD3 combination therapy administered after syngeneic or allogeneic pancreatic islet transplantation to inhibit recurrence of autoimmunity, and possibly allo-reactivity, and maintain insulin independence.

Pancreatic islets isolated from NOD-scid (syngeneic) or Balb-C donor mice (allogeneic) were transplanted under the kidney capsule of diabetic NOD (blood glucose level >300-400mg/dL) mice. The day after, successfully transplanted mice (normoglycemic: blood glucose level ~100 mg/dL) were treated with LV.InsB/anti-CD3, anti-CD3 mAb (1X 5μg) alone, or left untreated as control. LV.InsB/anti-CD3 treatment combined with syngeneic or allogeneic islets allowed stable normoglycemia in 50% and 40% of treated mice, respectively. Conversely, recurrence of diabetogenic responses rejected transplanted islets in two weeks in mice treated with anti-CD3 mAb alone or left untreated. AutoAg stimulation of splenocytes isolated from transplanted and treated with LV.InsB/anti-CD3 mice showed persistence of autoreactive T cells. Phenotypic analysis of T cells revealed that the frequency of FoxP3+ Tregs within CD4+ T cells of renal (RLN) and pancreatic (PLN) lymph nodes was increased, indicating that LV.InsB/anti-CD3 treatment induces in long-term normoglycemic transplanted NOD mice active suppression of autoimmune responses. These evidences confirmed the previously described mode of action of LV.InsB: Ag-specific FoxP3+ Tregs accumulating in the PLN and RLN keep under control autoimmune effector T cells at target sites of autoimmunity. By histological analyses of pancreas and transplant site we are investigating whether Ag-specific Tregs may also control allo-reactive effector T cells by a bystander regulation. The definition of novel strategies for the induction of Ag-specific tolerance combining autoAg expression in hepatocytes and tolerogenic compound such as anti-CD3 mAb, will be beneficial for the treatment of autoimmune diseases and possibly for the induction of transplantation tolerance.

202. Evolved AAV Capsids for Intramuscular Passive Vaccine Administration to Human Skeletal Muscle

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Skeletal muscle is ideal for passive vaccine administration as it is easily accessible by intramuscular injection. Indeed, recombinant adeno-associated viral (rAAV) vectors have begun being tested in clinical trials for intramuscular passive vaccination for HIV and influenza. However, greater human skeletal muscle transduction is needed for therapeutic efficacy than is possible with existing serotypes. To bioengineer capsids with therapeutic levels of transduction, we utilized a directed evolution approach to screen libraries of shuffled AAV capsids in pools of surgically resected primary human skeletal muscle cells from five patients. Six rounds of directed evolution were performed in various muscle cell types and evolved variants from each screen were validated against existing muscle-tropic serotypes. We show that evolved variants rAAV-NP22 and rAAV-NP66 have significantly increased primary human and rhesus skeletal muscle fiber transduction from surgical explants ex vivo, as well as in various primary and immortalized myogenic cell lines in vitro. In addition, we have demonstrated reduced seroreactivity compared to existing serotypes against normal human serum from 50 US donors. The advantage of rAAV-NP22 and NP66 vectors capable of transducing human skeletal muscle intramuscularly at such high levels would be to decrease passive vaccine dosing while still enabling therapeutic levels of antibody expression. This could bypass several hurdles to rAAV being an effective passive vaccine delivery tool: a) reduced potential for the generation of anti-antibody responses to rAAV-expressed antibodies like those shown for anti-HIV and anti-SIV broadly neutralizing antibodies; b) decreased likelihood for neutralizing anti-AAV capsid antibody binding since fewer circulating rAAV capsids would be present; c) reduced cost of vaccine production per patient; and d) reduced probability for capsid-specific T cell responses against transduced muscle fibers. These new capsids represent powerful clinical tools for human skeletal muscle expression and secretion of therapeutic quantities of antibodies from passive vaccines.

203. Exploiting the Pre-Existing Immunity to Adenoviruses for Cancer Immunotherapy

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Oncolytic adenoviruses have shown to be an excellent platform for cancer immunotherapy due to their ability to stimulate the immune system. Nevertheless, pre-existing immunity (PEI) to this vectors represents a recurrent concern for their use into the clinic. In addition, many combination therapies are expected to be studied due to the success of checkpoint inhibitors. However, how the PEI affects the efficacy of immunotherapies based on oncolytic adenoviruses is an aspect that requires a deeper understanding. Challenging the paradigm that PEI is hindering the efficacy of oncolytic viruses we aim at demonstrating that anti-viral T-cells play an important role into the overall success of virus-based immunotherapy. We studied the combination of our adenovirus-based cancer vaccine platform (PeptiCRAd) with checkpoint inhibitors (anti-PDL1) and demonstrated that mice treated with both combo-therapy experienced an increased median survival. In fact, Combination of PeptiCRAd and anti-PDL1 therapy increased the median survival of B16 melanoma bearing mice (43 days for Combo therapy versus 35 and 27.5 days for the anti-PDL1 and PeptiCRAd monotherapies, respectively). On day 28 all mice received an injection of PeptiCRAd targeting TRP2 and gp100 tumor antigens and were re-challenged with tumor cells. Interestingly, mice that were previously treated with PeptiCRAd + anti-PDL1 showed a slower tumor growth rate compared to previously treated only with anti-PDL1. Prompted by this observation, we tested if establishing pre-existing in mice would favour the subsequent oncolytic immunovirotherapy. To this end, we compared the efficacy of the same combination therapy between pre-immunized and näive mice. Surprisingly, PEI did not hinder the efficacy of immunovirotherapy, since the pre-immunized group showed a similar tumor growth and survival with näive mice. In addition, an higher degree of tumor rejection was observed among pre-immunized mice. ELISPOT assay confirmed the presence of a strong anti-viral adaptive immunity in mice. Finally, to elucidate the mechanisms throught with PEI could be exploited for oncolytic virotherapy, we immunized CD45.2 mice with adenovirus. Then, in order to asses the contribution of each anti-viral T-cell population to the anti-tumor response, we collected lymphoid organs and sorted CD8 and CD4 T-cells. We then transferred these cells to tumor bearing CD45.1 recipient mice, treated the mice with viruses and followed the tumor growth. Analysis of data and immunological studies from this mechanistic experiment will be presented at the ASGCT Annual Meeting.

204. Description of Structural Modifications of DNA Vector Encoded Monoclonal Antibodies (DMAbs) to Improve *In Vivo* Expression Levels After Intramuscular Injection and Electroporation

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Monoclonal antibodies (mAb) have broad applications for therapeutic use in humans, including cancer and infectious disease. It is estimated that there will be approximately 70 approved therapeutic antibodies on the market by 2020, with combined worldwide sales of $125 billion. DNA vector-encoded monoclonal antibodies (DMAbs) offer a means to generate *in vivo* mAbs by using electroporation (EP) to transfect skeletal muscle. In previous studies, we have demonstrated that DMAbs can achieve high serum levels and shown protection comparable to purified mAbs in flu and pseudomonas murine challenge models (Patel *et al.*, Elliott *et al.*). Working toward clinical application, we focused our efforts on further increasing the *in vivo* expression levels of DMAbs through formulation, administration, nucleotide and amino acid optimization. We present here two DMAb antibody modification strategies employing framework grafting and the development of scFv-Fc. Several DMAbs targeting emergent infectious diseases were selected as the foundation for these structural changes.

Multiple constructs were generated and screened for *in vivo* expression by ELISA. The partial graft method consists of replacing a portion of the variable light chain framework region from a poor expressor with that of a higher expressing DMAb. The new partial graft constructs showed increases approximately a log higher than the original DMAbs. Additionally, we tested scFv-Fc conversion, which intends to promote heavy chain - light chain pairing and tissue penetration. Converting DMAbs from a full length antibody to scFv-Fc resulted in peak expression of up to 4200 ng/ml, and some clones reaching a log fold increase compared to the original DMAb. Importantly, modifications made to the majority of DMAbs either retained or increased 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 protein structure modulation when designing DMAbs for clinical development.

205. Activation of DNA Pattern Recognition Receptors After Plasmid Electrotransfer in Multiple Tumor Cell Types

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Electroporation or electrotransfer is an effective delivery system for introducing plasmid DNA into cells and tissues. During this process, DNA primarily enters the cell via endocytosis, although some DNA may reach the cytosol. This DNA may be detected by endosomal, cytosolic, and nuclear DNA-specific pattern recognition receptors (PRRs) that have been described in many cell types. Activated PRRs induce the production of proinflammatory molecules and programmed cell death. Our previous studies on B16.F10 mouse melanomas demonstrated that electrotransfer of vector plasmid induced the production of several proinflammatory cytokines and chemokines including IFNβ, implicating the binding and activation of intracellular DNA-specific PRRs. Histologically, tumor necrosis independent of caspase-3 was observed. After DNA electrotransfer, levels of IFNβ mRNA and protein significantly increased in B16.F10 cells in culture. The mRNAs for several PRRs were present in these cells and the DAI/ZBP1, DDX60, and p204 mRNAs were significantly upregulated. DDX60 protein levels were coordinately upregulated. The goal of our study was to find out whether the effects observed in B16.F10 cells are widespread among tumor types. The electrotransfer of vector plasmid into TS/A murine mammary adenocarcinoma and WEHI 164 mouse fibrosarcoma cells induced IFNβ and TNFα mRNA and protein. In TS/A cells, the mRNAs of several PRRs (DAI/ZPB1, DDX60, DDX36, (not significant) and p204) were significantly upregulated, while in WEHI 164 cells, DAI/ZPB1, LRRFIP1, DDX60 p202 and p204 mRNAs were upregulated. Clearly, the repertoire of PRR mRNAs responding to DNA electrotransfer varies with the tumor cell type. The upregulation of DAI/ZPB1 mRNA was confirmed by Western blotting analysis in TS/A cells. Taken together, increased IFNβ and DNA sensor expression accompanied by cell death indicate that DNA electrotransfer activates intracellular PRRs in several tumor cell types, producing *in vitro* effects. *In vivo*, localized inflammation and induced cell death may contribute to cancer-targeted gene therapies delivered by electroporation.

206. Tracking and Imaging of Tumor Progression and Immune Function in a Preclinical Mouse Model

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Background: Prostate cancer is a major cause of death in men around the world. Despite a variety of treatments, disease progression and metastases still occur in most cases. Given the promising effect of combination with immunotherapy for prostate cancer, the construction of an immunocompetent mouse model for simultaneous monitoring of tumor volume, tumor biomarker and immune cell functions, would be useful for further understanding the mechanism of tumor progression and immune regulation. **Methods:** Through genetic engineering techniques, a new cell line, RM9-Luc-pIRES-KLK3 was constructed. The cells were inoculated into immunocompetent mice of strain C57BL/6 via dorsal flank, dorsolateral prostate and tail vein to obtained subcutaneous model, orthotopic model and metastasis model, respectively. Tumor volumes, non-invasive imaging and prostate-specific antigen (PSA) were evaluated. In the metastasis models, either anti-CTLA-4 antibody or PBS was administered to the tumor bearing mice, and the status of circulating immune cells was assessed by flow cytometry. **Results:** The new cell line, RM9-Luc-pIRES-KLK3 was successfully constructed and steadily expressed PSA and Luc, which were confirmed by Western blotting and bioluminescence detection in vitro. The level of expression was positively correlated with cell counts. Three days after injection, RM9-Luc-pIRES-KLK3 cells grew readily in the mice and the tumors could be detected by IVIS imaging system from then on. Four days later, PET scan was conducted to confirm the lesions. The intensity of bioluminescence imaging in coronal section and FDG uptake in sagittal slices of PET imaging were totally overlay. Comparing with PBS treated mice; MDSCs and T regs in peripheral blood were significantly decreased in the tumor bearing mice treated with anti-CTLA-4. Meanwhile, the proportion of CD44+CD62− effector and memory T cells on CD3+CD8+ cells were significantly increased by >2-3 times after CTLA-4 blockade compared with the control treatment, as well as IFNγ and TNFα. **Conclusion:** The presented models were ideally suited for real-time tracking of drug response and imaging of tumor progression and immune function. In comparison with traditional methodologies, this biomarker/imaging-based approach could lead to improved, early, and sensitive assessment of tumor status.

207. Analytical Validation of Real-Time PCR Method for the Quantification of HSV-2 Plasmid DNA Vaccine in Rat and Monkey Tissues

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Vaccines based on plasmid DNA have been prepared for a number of infectious agents. They stimulate both the antibodies and cell-mediated components of the immune system. Herpes Simplex virus 2 (HSV-2) is a large DNA virus that is highly prevalent in human populations in many parts of the world and is the most common cause of genital ulcer disease worldwide. As a part of preclinical safety evaluation of DNA vaccine development, it is necessary to investigate biodistribution/ persistence of plasmid DNA in animals using a sensitive detection method such as quantitative real-time PCR (qPCR) technique. The present study was conducted to establish an analytical validation method by qPCR for quantification of a plasmid DNA vaccine targeting HSV-2 (HSV-pDNA) in rat and monkey tissue samples. The validation was conducted to evaluate for specificity, linearity, accuracy, and precision. There were no interfering reacts with the targeted sequence between true positive and negative control (no template control) samples. The correlation coefficient of HSV-pDNA were 0.998 and 0.9963 ($1/x^2$ weighted) in rat and monkey tissue genomic DNA (gDNA), respectively. The limit of detection for HSV-pDNA was also 10 and 300 copies in 1 ug of tissue gDNA, respectively. Furthermore, the accuracy and precision were verified by intra- and inter-assay analysis. Therefore, the analytical method was quantitative with good linearity, accuracy and precision for HSV-pDNA. In conclusion, the highly specific and sensitive qPCR analytical method for HSV-pDNA was well-established in this study and would be also useful for further preclinical biodistribution/ persistence studies of DNA vaccines in animals.

208. InvossaTM (TissueGene-C) Induces an Anti-Inflammatory Intra-Articular Environment in a Rat MIA Model via Macrophage Polarization

Hyeonyoul Lee[1], Kyoungbaek Choi[1], Heungdeok Kim[1], Daewook Kim[1], Hyesun Lee[1], Yunsin Lee[1], Bumsup Lee[1], Sujeong Kim[1], Myung Chul Lee[2], Heonsik Choi[1]

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Background Invossa™ (TissueGene-C) is a novel cell and gene therapy for osteoarthritis (OA). Late stage clinical trials demonstrated that TissueGene-C improved pain, function, and cartilage structure in patients with OA. However, the exact mechanism through which TissueGene-C works has not been determined. Here we explore the hypothesis that TissueGene-C induces an anti-inflammatory response via M2 macrophage polarization. We tested this hypothesis in a rat, mono-iodoacetate (MIA) model of OA. **Result** Pain relief was noted on day 7 and maintained until day 56 after TissueGene-C treatment. Regeneration of cartilage was observed in animals treated with TissueGene-C. Cytokine expression profiles showed that TissueGene-C increased IL-10 levels in synovial lavage fluid after 4 days of treatment. Furthermore, TissueGene-C increased the number of cells positive for arginase 1, a marker of M2 macrophages, which play a critical role in wound healing and possesses anti-inflammatory regulatory functions. On the other hand, the number of cells positive for CD86, a marker of M1 macrophages, which play key roles in acute inflammation and secrete high levels of inflammatory cytokines in the synovial membrane, was decreased in the TissueGeneC treated animals. Quantitative RT-PCR analysis also showed that M2 macrophage-related markers were highly up-regulated in the synovial membranes from TissueGene-C treated animals. **Conclusion** The anti-inflammatory cytokine IL-10 and M2 macrophages were highly elevated in TissueGene-C- treated knee joints in a rat MIA model indicating that TissueGene-C treatment induced the anti-inflammatory response. This mechanism may contribute to the pain and cartilage improvement noted in patients treated with TissueGene-C in the clinical trials.

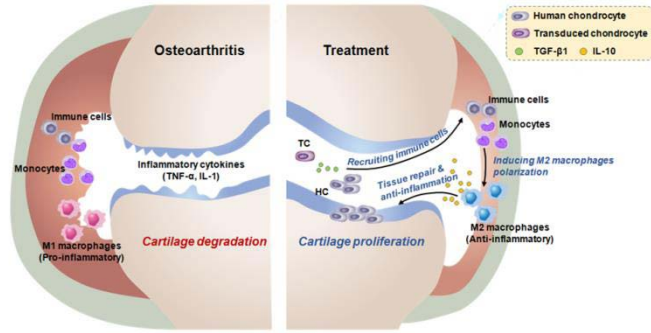

Figure. A proposed mechanism of INVOSSA™.

Musculo-skeletal Diseases I

209. rAAV-Microdystrophin Transduction with MSCs Pre-Treatment of Canine X-Linked Muscular Dystrophy Improved Transgene Expression and Their DMD Phenotype

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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 effective to improve pathogenesis of animal models of DMD. However, we have 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 rAAV vector and transgene expression. MSCs regulate various inflammatory diseases including GVHD by virtue of their immunosuppressive effects. **Methods:** Bone marrow derived MSCs and rAAV9-Luciferase or rAAV9-microdystrophin were intravenously injected into the normal or $CXMD_J$ dog at 8 weeks old. Seven days after injection, MSCs were systemically injected again. At 8 days after 1st injection, rAAV9-Luciferase or rAAV9-microdystrophin were intravenously injected into the same dog. To examine the immune response against rAAV, purified canine peripheral leukocytes were exposed to rAAV9, and then IFN-γ expression was analyzed using qRT-PCR. Expressions of transgene in skeletal muscles of the rAAV-Luciferase or rAAV-microdystrophin transduced animals were confirmed by immunohistochemistry. **Results:** Administration of rAAV following MSCs treatment resulted in higher expression of transgene (Luciferase or microdystrophin) at the skeletal muscle, compared to the rAAV transduction alone. Expression of IFNγ in the purified peripheral blood leukocytes after the rAAV exposure were not enhanced in the rAAV 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 same age. **Conclusion:** Our results demonstrate that rAAV injection with MSCs pre-treatment improved expression of rAAVderived transgene in dogs. This strategy would be effective approach to analyze the expression and function of transgene *in vivo*. These findings also support the future feasibilities of rAAVmediated protein supplementation strategies to treat DMD.

210. Reduction of Autophagic Accumulation in Pompe Disease Mouse Model Following AAV Gene Therapy

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Pompe Disease (PD) is a fatal metabolic disorder caused by mutations in the *GAA* gene leading to a deficiency in acid alpha-glucosidase (GAA) and affects 1 in 40,000 births. Clinically, this lysosomal storage disorder presents with cardiomegaly and skeletal muscle weakness, leading to cardiorespiratory failure. GAA is responsible for the breakdown of glycogen in the lysosome, an important energy source for striated muscle and neurons. Currently, the only approved treatment for PD is enzyme replacement therapy (ERT). While ERT has increased patient survival, there are several limitations including treatment cost and the inability of the enzyme to cross the blood-brain barrier and breakdown lysosomal glycogen deposition within the central nervous system. ERT therapy is dependent upon receptor-mediated endocytosis of the exogenous enzyme; much of which remains in amphisomes resulting in insufficiently trafficking of GAA to the lysosome. These issues illustrate the need for an alternative treatment. We propose that endogenous production of GAA enzyme mediated by adeno-associated virus (AAV)-delivery of the *GAA* gene will improve targeting of GAA to the lysosome and reduce the overall dysregulation of vesicular systems. In this study, we performed intravenous delivery of AAV9-DES-coGAA to 12-week old $Gaa^{-/-}$ animals at three doses ($1x10^{11}$ vg/kg, $1x10^{13}$ vg/kg, and $1x10^{14}$vg/kg) and a vehicle-control group. Previous research has revealed that cellular dysregulation due to lysosomal storage of glycogen is amassed by this age prior to onset of the physiological phenotype of PD in the $Gaa^{-/-}$ murine model. Following AAV or sham injection, cardiac and skeletal muscles were harvested for biochemical and histological analyses. GAA activity assays demonstrate that therapeutic levels of enzyme activity are not obtained in skeletal muscle with $1x10^{11}$ vg/kg and $1x10^{13}$ vg/kg dose, but were when a dose of $1x10^{14}$ vg/kg was administered. Vacuolization of fibers, observed through Hematoxalin and Eosin staining, showed significant decrease when therapeutic levels of GAA were produced. Immunofluorescence detecting LAMP1 and LC3, indicate that vacuoles are lysosomes and autophagosomes. Total levels of autophagy-associated proteins, such as LAMP1, LC3-I, LC3-II, Beclin 1, and p62 decrease indicating an improvement in autophagic accumulation. Cross-sectional area of fibers in the treated muscles was increased, trending toward the sizes observed *wildtype*. The levels of each protein and other parameters evaluated indicate that while most fibers respond favorably, the various fiber types (I, IIa, IIb) the mechanism by which they are corrected is different, an avenue being further pursued. Taken together, particularly in Type IIb muscle fibers, which are resistant to ERT, there is a significant improvement in the cellular pathology of PD mouse model skeletal muscle, which is likely to correlate to the functional advancements documented by others following AAV-mediated gene therapy.

211. Long-Term Characterization of In Vivo Genome Editing in a Mouse Model of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a genetic disorder caused by gene deletions, duplications, or nonsense mutations leading to the loss of the essential musculoskeletal protein dystrophin. Patients experience muscle wasting, loss of ambulation in the teen years, followed by premature death by 30 years of age. *In vivo* genome editing has emerged as a potential approach to correct the genetic mutation that causes DMD. The potential advantage of gene-editing over gene-replacement strategies is the possibility for permanent correction of the endogenous gene. We and others have reported the use of genome editing using *Streptococcus pyogenes* Cas9 (SpCas9) or *Staphylococcus aureus* Cas9 (SaCas9) for deletion of exon 23 in the mdx mouse model of DMD. Adeno-associated virus delivering paired RNA-guided nucleases flanking exon 23 in the dystrophin gene corrected the reading frame and restored functional dystrophin protein expression. These reports also showed improved muscle biochemistry and muscle function from four to eight weeks after treatment (*1-3*). However, further characterization of the persistence of gene editing, off-target activity, and immunogenicity is still needed. In addition, Cpf1 derived from *Lachnospiraceae* (LbCpf1) has been adapted for genome editing (*4*) and may find additional utility in repairing the dystrophin gene.

Systemic administration of AAV8 containing SaCas9 and gRNAs in adult and neonatal mdx mice edited the dystrophin gene in multiple skeletal muscles and cardiac muscle leading to dystrophin restoration by immunofluorescence (IF) eight weeks after the single treatment. One year after a single administration, dystrophin expression is sustained in cardiac muscle, however, a decrease in dystrophin expression was observed in the skeletal muscle by IF. To make quantitative comparisons of genome editing efficiency over time, more quantitative measures will be employed including next-generation sequencing and digital drop PCR. In addition to SpCas9 and SaCas9 we have also adapted LbCpf1 for deletion of exon 51 in patient-derived myoblasts leading to restoration of a frame-corrected dystrophin transcript. Ongoing work will draw comparisons between gene-editing strategies using SaCas9 and LbCpf1. Additional work will seek to improve the long-term efficiency and safety of *in vivo* genome editing including the use of muscle-specific promoters, optimization of vector transgene, and characterization of AAV serotypes.

212. RNA-Seq Analysis of Canine X-Linked Myotubular Myopathy Muscles Before and After Gene Therapy

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X-linked myotubular myopathy (XLMTM) is a severe congenital myopathy resulting from mutations in the myotubularin gene (*MTM1*). These last years, gene therapy using recombinant adeno-associated virus (rAAV)-derived vectors has emerged as a promising therapeutic approach. Using the canine model of XLMTM, we have conducted multiple studies to establish the minimally effective dose of vector and to monitor the long-term efficacy of gene therapy. Recently, we have recorded significant therapeutic benefits in two XLMTM dogs up to 4 years post rAAV infusion, and human trials of this vector are planned to start in 2017. Massively parallel transcriptome analytics, such as RNA-Seq, are becoming increasingly standardized, and offer great potential as tools for analyzing the consequences of rAAV-mediated gene therapy. To gain insight into the mechanisms by which gene therapy improves the canine XLMTM phenotype, and further our understanding of the pathology in this model, we analyzed the muscle transcriptome of wild-type (WT) dogs and XLMTM dogs treated with rAAV gene therapy or placebo. First, we used RNA-Seq to characterize the transcriptional consequences of *MTM1* deficiency in canine muscles. We found dysregulations of genes involved in muscle development, myofibril structure, excitation-contraction coupling, and key signaling pathways. In addition, gene ontology analysis allowed us to pinpoint original features of the XLMTM pathology, including the reactivation of developmental genes, and multiple dysregulations of genes participating in the structure and function of the extracellular matrix and adipose tissue. Next, we focused our analysis on XLMTM dogs whose phenotype was successfully rescued after rAAV gene therapy. A similar RNA-Seq analysis on these muscle samples indicates that as rAAV dose increases, the transcriptome of treated dogs becomes more similar to those of WT animals and less like untreated XLMTM counterparts, proving that gene therapy leads to a dose-dependent transcriptional remodeling in muscle cells. Likewise, vector-derived *MTM1* sequence tag counts correlate with injected dose and therapeutic efficacy. Finally, we identify and distinguish rAAV-sensitive, rAAV-resistant, and rAAV-induced transcripts, the further characterization of which could help refine future therapeutic approaches and improve the safety and efficacy of XLMTM gene therapy. In conclusion, our data show that RNA-Seq is a powerful platform for analyzing rAAV-transduced tissues, and argue for its continued development into validated assays for monitoring neuromuscular gene transfer clinical trial samples.

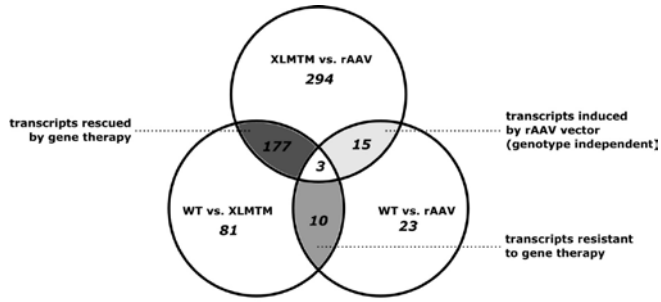

Figure: Venn diagram representing the number of differentially expressed transcripts between Wild Type dogs (WT) and dogs affected by X-linked myotubular myopathy, treated with a therapeutic dose of recombinant adeno-associated vector (rAAV) or placebo (XLMTM).

213. Age-Dependent Response to FKRP Gene Transfer

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Loss-of-function mutations in the Fukutin-related protein (*FKRP*) gene cause limb-girdle muscular dystrophy type 2I (LGMD2I) and other forms of congenital muscular dystrophy-dystroglycanopathy that are associated with glycosylation defects in the α-dystroglycan (α-DG) protein. Systemic administration of a single dose of recombinant adeno-associated virus serotype 9 (AAV9) vector expressing human *FKRP* to a mouse model of LGMD2I at various stages of disease progression was evaluated. Results demonstrate rescue of functional glycosylation of α-DG and muscle function along with improvements in muscle structure at all disease stages versus age-matched untreated cohorts. Nevertheless, mice treated in the latter stages of disease progression revealed a decrease in beneficial effects of the treatment. The results provide a proof-of-concept for future clinical trials in *FKRP*-related muscular dystrophy patients and demonstrate that AAV-mediated gene therapy can potentially benefit patients at all stages of disease progression, but earlier intervention would be highly preferred.

214. Gaining Insight into FSHD Through Localization of DUX4 Protein in Human Biopsies

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Understanding disease pathology for facioscapulohumeral muscular dystrophy (FSHD) to inform and test therapeutic approaches has been challenging due to the lack of a representative mouse model of this dominant disease. The genetic mutation causing FSHD is a DNA macrosatellite repeat contraction that results in expression of the gene *DUX4*. Modeling FSHD through DUX4 expression is problematic due to the sensitivity of mouse muscle to the damaging effects of DUX4 expression. While transgenic models are under development from many groups, our laboratory has used AAV to deliver the *DUX4* gene, with its cognate promoter, to adult mouse muscle. Prior to this study the DUX4 protein has never been identified *in vivo*, either in an animal model or in human muscle biopsies. To investigate the relationship of DUX4 protein expression to FSHD pathology we analyzed cryosections from 15 FSHD and 6 unaffected control needle biopsies for the presence of DUX4 protein using immunohistochemical and confocal imaging methods, where the examiner was not informed of disease status. In a second similar ongoing study, we probed 120 of 150 total additional larger, muscle open biopsy cryosections from FSHD and control subjects. Of 141 cryosections examined so far from both studies we found 4 isolated DUX4 positive nuclei and a regional cluster that was later revealed to be FSHD-muscle derived. Additionally, needle biopsies have been collected from FSHD patients and controls to conduct progressive screenings of larger regions of multiple biopsies. The infrequent occurrence of DUX4 primarily in myonuclei suggests brief focal protein expression may be sufficient to cause disease. This finding is consistent with DUX4 protein expression in patient myotubes grown *in vitro*, where DUX4 protein is typically detected at a frequency of 1 in 5000 cells. The incidence of expression also can be correlated with dose of AAV-DUX4 delivered to the TA of adult wild-type mice and the level of myofiber death in our mouse model. Reducing AAV-DUX4 dose in intramuscular injections produced barely detectable levels of DUX4 protein. Low dose AAV-DUX4 of $2x10^9$ vector genomes produced progressive, persistent muscle damage, inflammation and adipose accumulation as in the human condition. Delivery of AAV-RNAi vectors targeting DUX4 mRNA greatly reduced the effect of DUX4 expression and supports DUX4 as the cause of muscle damage. The AAV-DUX4 model is a powerful, tunable model for studying disease mechanism, guiding investigation of the role of DUX4 expression in FSHD, and for identifying and testing promising candidate therapies.

215. AAV-Mediated Transfer of FKRP Shows Therapeutic Efficacy in a Murine Model of Limb-Girdle Muscular Dystrophy Type 2i, but Requires Tight Control of Gene Expression

Isabelle Richard

Genethon, Evry, France

Limb Girdle Muscular Dystrophies (LGMD) type 2I, a recessive autosomal muscular dystrophy, is caused by mutations in the Fukutin Related Protein (FKRP) gene. It has been proposed that FKRP, whose function remains unclear, is a participant in α-dystroglycan (αDG) glycosylation, which is important to ensure the cell/matrix anchor of muscle fibers. A knock-in mouse model of LGMD2I was generated to express the most frequent mutation (L276I) encountered in patients. The introduction of the mutation did not alter the expression of FKRP, neither at transcriptional nor at translational levels, but did alter its function since abnormal glycosylation of αDG was observed. In this model, skeletal muscles were functionally impaired from 2 months of age and a moderate dystrophic pattern was evident by histology starting from 6 months of age. Gene transfer with a rAAV2/9 vector expressing *Fkrp* restored the biochemical defects, corrected the histological abnormalities and improved the resistance to eccentric stress in the mouse model was obtained. However, injection of high doses of the vector induced a decrease of αDG glycosylation and laminin binding. Finally, we showed that intravenous injection of the rAAV-*Fkrp* vector into a dystrophic mouse model suffering of dystroglycanopathy due to skeletal muscle-specific Fukutin (*Fktn*) knock-out caused toxicity. The dose-dependent worsening of the dystrophic phenotype suggests requirement for a precise control of its expression.

216. Nonhuman Primate Safety and Potency of an AAV Vector for XLMTM Produced by Transient Transfection at 500L

Angelica Phillips, Archana Belle, Jianjun Guo, Jennifer Ton, Tim Stinchcombe, Ana Buj-Bello, John T. Gray

Audentes Therapeutics, Inc., San Francisco, CA

AAV vectors delivering a desmin promoter-driven *MTM1* cDNA have demonstrated robust efficacy in both mouse and dog models of X-Linked Myotubular Myopathy (XLMTM). Consistent with other neuromuscular diseases (NMD), the doses shown to reverse the pathology exceed 10^{14} vg/kg. Although the potential for these and other AAV therapies to transform the treatment of NMD has been established, the manufacturing challenges created by high dose systemic AAV gene therapy remains a major issue for the field, and it is critical to demonstrate that products manufactured at large scale maintain adequate potency and quality. We have established a new clinical manufacturing facility with capacity to meet worldwide demand for multiple orphan NMD, and here report the results of an IND-enabling nonhuman primate safety study of AT132 (AAV8-Des-hMTM1) made in that facility using transient transfection of suspension HEK293 cells at the 500 L scale.

Three infant macaques were given 8×10^{14} vg/kg of AT132 i.v., and along with 2 control animals were monitored for 8 weeks prior to necropsy and assessment of vector biodistribution and pathological endpoints.

Clinical pathology, including serum markers of liver, muscle, and cardiac tissue damage, and cardiac function (ECG) did not exhibit significant impact from AT132 administration. One AT132 treated animal exhibited symptoms of an iron deficiency anemia which can be detected in infant macaques on milk diets and was not considered test article related. Consistent with this finding, this animal exhibited a pale liver and mild hypocellularity of the marrow on necropsy. Minimal to moderate monocytic infiltration was observed in 2 of the 3 treated NHPs in several tissues including the skeletal muscles, the intended target of the therapy.

Immunologically, all animals had undetectable AAV8 neutralizing antibodies (NAbs) in serum at screening, although 2 animals seroconverted just prior to dosing. These two animals, with NAb titers of 1:40, were split between control and treatment cohorts. After dosing with AT132, both NAb and total IgG specific for AAV8 capsid developed in all dosed animals. MTM1 protein-specific antibodies and T-cell ELISPOT signals in response to both MTM1 and AAV8 capsid peptides were not observed at any timepoint.

To assess the potency of the AAV product in NHPs, vector genomes per diploid cell genome (vg/dg), mRNA, and MTM1 protein levels were assessed in multiple tissues. Notably, although the AAV8 seropositive animals had reduced vector genomes in tissues, all treated animals exceeded 100 vg/dg in the liver, and 15-50 vg/dg in skeletal and cardiac muscle. MTM1 protein expression by western blot revealed levels 10-50-fold over those found in control animals in skeletal muscle, a finding corroborated by RNA-Seq data analysis, and 10-50 fold higher than the levels associated with complete rescue of the XLMTM canine model. Despite high copy transduction in the liver, MTM1 protein levels in that tissue were in the normal range.

In summary, AT132 produced at 500L scale demonstrated an encouraging safety profile and high potency, providing strong justification to advance AT132 into clinical testing for XLMTM patients. Furthermore, the ability to rapidly manufacture large quantities of highly potent AAV vectors should accelerate development of novel therapies for a wide variety of NMD.

217. 3D Printed Hyperelastic Bone Scaffolds and Regional Gene Therapy. A Multidisciplinary Approach to Bone Healing

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Introduction: Regional gene therapy has the potential to heal complex bone defects through placement of BMP-2-transduced cells on a carrier. However, the impact of the cell carrier on bone healing in regional gene therapy has received limited attention. Three-dimensional (3D) printing allows for precise control of biologic properties and micro-architecture of carrier scaffolds, which can facilitate the efficacy of this treatment regimen. The purpose of this study was to evaluate the viability of human adipose-derived stem cells (ASCs) transduced with a lentiviral vector to overexpress BMP-2. The transduced cells were loaded on to a 3D printed scaffold comprised of a novel material, Hyperelastic Bone (HB) **Methods:** Human ASCs were transduced with a lentiviral vector (LV-BMP-2) carrying the cDNA for BMP-2. These transduced cells were then loaded on to 3D printed Hyperelastic Bone (HB) scaffolds. HB is a new, synthetic, osteoregenerative biomaterial, which is composed of 90% hydroxyapatite, yet is mechanically elastic. In vitro BMP-2 production of transduced ASCs loaded on to the HB scaffold was assessed using ELISA analysis in triplicate. In addition, muscle pouches in the hind limbs of nude mice were implanted with $2x10^6$ ASCs transduced with LV-BMP-2 (Group I), LV-GFP (Group II), or ASCs alone (Group III). We also implanted empty HB scaffolds (Group IV). Three mice were implanted per group. Radiographs were taken at 2 and 4 weeks. Muscle pouches were dissected out at 4 weeks and underwent histologic analysis following standard protocols. **Results:** BMP-2 production on the HB scaffold at 24 hours was abundant, and continued to increase after 1 and 2 weeks of culture (Table 1). Robust bone formation was noted at 2 and 4 weeks in Group I on plain radiographs (Figure 1); no bone formation was noted in Groups II-IV. Histologic analysis demonstrated that all groups had healthy scaffold integration with host tissue, including vascularization, and extracellular matrix penetrating the HB material. Only group I demonstrated evidence of woven bone formation (Figure 2). **Conclusions:** Custom 3D printed HB scaffolds may serve as an effective carrier for transduced cells to promote bone repair. The ability to 3D print HB scaffolds contoured to fit specific anatomic defects and its compatibility with regional gene therapy has significant clinical potential for bone healing.

In vitro BMP-2 production	
Time Point	**BMP2 production (ng/ 24h/10^6 cells)**
24 hours	21.09
1 week	61.75
2 weeks	76.28

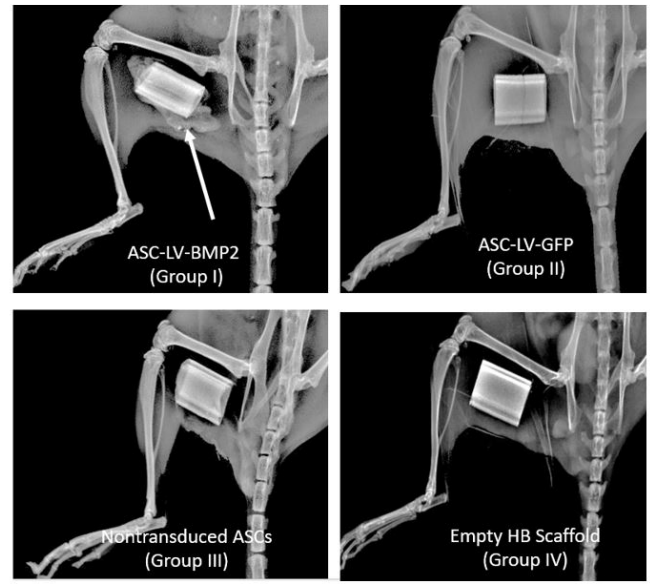

Figure 1. Plain radiographs taken at 2 weeks after implantation of the muscle pouch with the Hyperelastic Bone Scaffold. There is evidence of robust bone formation (white arrow) in the group implanted with adipose derived stem cells (ASCs) transduced with a lentivirus (LV) carrying the cDNA for BMP-2 (Group I, top left). There is no evidence of bone formation in Groups II-IV.

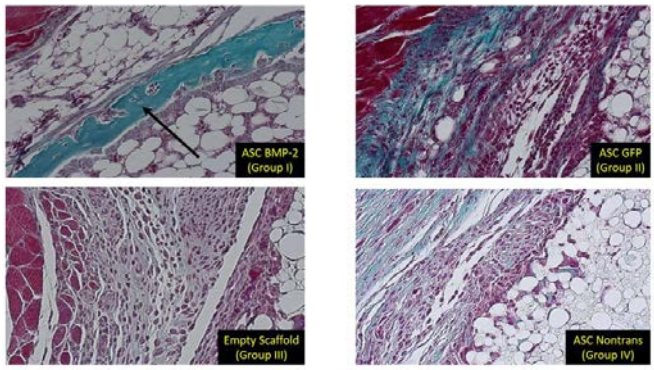

Figure 2. 20x histology images stained with Masson's trichrome. The histological slices represent the same area of each muscle pouch at the interface between the exterior of the Hyperelastic Bone scaffold and native muscle. In Group I there is evidence of woven bone formation (black arrow). There is no evidence of bone formation in Groups II-IV.

218. Therapeutic Capacity of rAAV-Micro-Utrophin in *mdx*^{4cv} Skeletal Muscles

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Duchenne muscular dystrophy (DMD) is a severe muscle wasting disorder caused by dystrophin gene mutations. Utrophin is a dystrophin paralogue that can prevent necrosis in the *mdx* mouse DMD model. We utilized an expression plasmid containing a miniaturized M-creatine kinase regulatory cassette (CK8e) and a rationally designed micro-utrophin$^{\Delta R4\text{-}21/\Delta CT}$ (μUtrn) cDNA to accommodate the limited capacity of recombinant adeno-associated viral vector (rAAV). Delivery of rAAV6-CK8e-μUtrn to young *mdx*^{4cv} mice led to μUtrn expression in ~90% of muscle fibers 2 weeks after treatment. However, examination of the μUtrn treated muscles at later timepoints (3-6 months) revealed a progressive deterioration of dystrophic pathology. Preliminary evidence suggests a lack of cooperativity between μUtrn and full-length utrophin may contribute toward diminished therapeutic benefits as 1) μUtrn was gradually reduced in myofiber types 1a, 2a, & 2d/x that display upregulated full-length utrophin, 2) μUtrn was found in 2a, 2d/x, and 2b fiber types equally in *mdx*:utrophin double knockout muscles, and 3) CK8e-μdystrophin$^{(\Delta H2\text{-}R19,\Delta R20\text{-}R23,\Delta CT)}$ was maintained in most *mdx* fibers for >7 months. Nevertheless, μUtrn expression was sufficient to improve specific force and partially protect the muscles from contraction-induced injury. Importantly, μUtrn partially restored the folding within the myotendinous junctions without leading to tears in the junctions or ringed myofibers, such as were found previously with expression of an early microdystrophin$^{(\Delta R4\text{-}R23/\Delta CT)}$ design. Furthermore, μUtrn partially prevented the fragmentation of neuromuscular junctions, restored the postsynaptic fold length, but not the number of postsynaptic folds. These results raise potential challenges for the treatment of DMD with rAAV-μUtrn.

219. Skeletal Muscle Function and Morphology Following Chronic ChR2-Based Stimulation

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Physical inactivity, cancer cachexia, and neuromuscular disease result in skeletal muscle atrophy and weakness. The effects of exercise on skeletal muscle regulation during these conditions appears to be dependent on the mode, frequency, and duration of exercise stimulus. However, when atrophy and contractile dysfunction reach advanced stages adherence and benefit of exercise regimens often become limited. As an alternative to exercise-based stimulation, optogenetics may serve as a non-invasive method of targeted neuromuscular activation and ultimately enhance or maintain skeletal muscle regulation and function. 129SVE animals were randomly assigned to control-sham (CON) or AAV-ChR2-tdTomato (AAV). CON and AAV animals received a single injection of vehicle or AAV in the left tibialis anterior (TA), respectively. AAV animals received LED-based light stimulation of the TA for 30 minutes/day for 10 days. Two days after the final stimulation bout, animals were euthanized to assess body and TA weight, AAV transduction efficiency, cross-sectional area (CSA), and mRNA analysis of genes associated with NMJ stability and skeletal muscle regulation. No significant effect was detected in body weight between CON and AAV animals. AAV administration resulted in ~46% transduction of myofibers as assessed by detection of tdTomato. AAV animals demonstrated contraction of the TA upon LED stimulus throughout the training regimen. Elevated expression of genes associated with NMJ formation and stability were observed in AAV animals. Overall TA muscle wet weight was significantly increased in AAV treated muscle when compared to the contralateral leg. Comparison of the CSA ratio between the injected and contralateral TA revealed an approximate 45% increase in animals receiving AAV. AAV mediated expression of ChR2 in skeletal muscle permits chronic LED light-based activation of skeletal muscle. Our data suggests LED stimulation of ChR2 enhances the NMJ profile and augments overall muscle mass and CSA. This effect is localized to the site of AAV administration and may serve as an alternative to exercise based therapy for conditions which result in muscle atrophy.

220. High Incidence of Liver Cancer in FKRP-Mutant Mouse Models

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Mutations in fukutin-related protein (FKRP) cause several common forms of muscular dystrophies ranging from the mild limb girdle muscular dystrophy 2I (LGMD2I) to severe conditions like congenital muscular dystrophy (MDC1C), and Walker-Warburg syndrome (WWS). Emerging evidence has shown that FKRP serves as an essential ribitol-phosphate transferase in glycosylation of alpha-dystroglycan, which is involved in a number of processes including basement membrane assembly, sarcolemma stability, and cell survival. Since the

initial discovery of FKRP in 2001, FKRP-related diseases have been categorized as neuromuscular disorders. However, we have observed a high incidence of liver cancer occurrence in old (>18-months-old) FKRP-mutant mouse models in our study. Specifically, 8 out of 13 of compound heterozygous E310^{stop}/L276I mice (an intermediate disease phenotype model) developed liver cancer (incidence rate of 61.5%). Additionally, 3 observed old homozygous L276I^{neo+} mice (a mild disease phenotype model) developed liver cancer (incidence rate of 100%). To note, 7 out of 8 mice treated with AAV-FKRP vector containing liver microRNA targeting (miR122T) sequences (for down-regulating liver expression) developed liver cancer (incidence rate of 87.5%). We did not see any liver cancer cases in wild-type control mice (n=5, incidence rate of 0%). H&E staining was performed on tumor tissues for morphology examination in addition to immunofluorescent staining against liver cancer marker AFP (alpha-fetoprotein). H&E staining displayed a typical hepatocellular adenoma and carcinoma, and the intense AFP expression revealed by immunofluorescent staining further confirmed that the observed tumors were liver cancer cells. Our study indicates that FKRP has essential roles in maintaining liver homeostasis at older age and an appropriate amount of FKRP expression in the liver is necessary for treatment of FKRP-related diseases in mouse models.

221. Utilizing RNA Guided Endonucleases to Develop an Osteoblast Like Cell Line to Study Collagen Trafficking and Quality Control

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Although it is the most abundant protein in the human body, intracellular trafficking of type I collagen, as well as other collagen types, is poorly understood. Pathological accumulation of collagen in the ER and disruptions in collagen secretion have been implicated in a variety of connective tissue disorders, yet the molecular mechanisms underlying these defects and the resulting cell stress response remain largely unclear. Live-cell imaging revealed multiple surprising features of trafficking of transfected fluorescently-tagged collagen chains. For instance, our laboratory observed unexpected composition of vesicles that transport properly folded collagen from the ER to Golgi as well as redirection of misfolded collagen from the secretory to autophagy-mediated degradation pathway at ER exit sites. However, the transfections themselves caused significant over expression of collagen leading to additional cell stress and abnormal collagen accumulation in the ER. In order to avoid potential artifacts associated with transient transfection and uncontrolled expression of collagen, we propose using RNA guided endonucleases to fluorescently tag the endogenous *col1a2* gene in MC3T3 mouse osteoblast line. FRT recombination sites will be placed along the length of *col1a2* to enable switching of the fluorescent tag as well as to introduce mutations that mimic osteogenesis imperfecta (OI). Such mutations cause moderately severe to lethal bone fragility and skeletal deformities in 1:20,000 births. The mechanisms of bone pathology in OI are still debated and there is no effective treatment for this disorder. An osteoblast cell with fluorescently tagged a2 chain of type I collagen, the sequence of which can be readily manipulated, will provide a unique model for live-cell imaging studies of normal and mutant collagen trafficking and processing by the cells as well as delineation of mechanisms of cell stress response to OI mutations. This line will allow us to investigate how collagen is trafficked through the cell, how misfolded collagen is trafficked into autophagosomes, and how the type of mutation and location of the mutation affect these trafficking events.

Neurologic Diseases (including Ophthalmic and Auditory Diseases) I

222. A Phase I/II Trial of Gene Therapy for an Inherited Disorder of Monoamine Neurotransmitter Deficiency

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Dopamine, serotonin, epinephrine, and norepinephrine are monoamine neurotransmitters produced through the activity of aromatic L-amino acid decarboxylase (AADC). An inherited deficiency of AADC activity leads to severe motor, autonomic, and cognitive dysfunction in infants and children. We have previously studied intraputaminal injection of an AAV2-hAADC vector, named as AGIL-AADC currently, in a compassionate use program, and demonstrated improvements in the motor function of patients with AADC deficiency. This phase I/II trial enrolled 10 AADC deficiency patients (1.7 to 8.4 years) with bilateral intraputaminal injection of the same vector. All stereotactic surgeries and vector injections were well tolerated. Patients started to move their arms and mouths 2-3 weeks after gene transduction, and new motor skills were observed 2-3 months later. At 12 months after gene transduction, all but one patient had improvements in motor scales, showed increase in cerebral spinal fluid neurotransmitter concentrations, and increase in tracer uptake in FDOPA PET. Anti-AAV2 antibody titers rose in all patients but titers decreased a few months after gene transduction. There were no signs of cerebral or systemic immune reaction during the follow up period. Adverse events related to treatment were generally well tolerated, including events associated with the surgery or transient post-gene transduction dyskinesia. One patient died of influenza B encephalopathy 10 months after gene transduction, but his 9-month motor scales had shown improvement. Preliminary evidence showed more substantial improvements in motor and cognitive function in the youngest patients treated. In conclusion, AAV2-hAADC gene therapy shows promise as a potential treatment for an inherited brain neurotransmitter deficiency, and treatment at a younger age may be associated with a better outcome.

223. Anc80 as a Novel Gene Transfer Tool for the Central Nervous System After Peripheral Delivery

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Gene therapy applied to neurodegenerative diseases is an evolving field that relies on the development of efficient and safe gene delivery systems. Among others, adeno-associated virus (AAV) vectors have shown the greatest promise in the treatment of genetic or acquired diseases of the central nervous system, being generally well-tolerated and highly efficient at transducing neural cells. Importantly, the characterization of novel AAV serotypes that can cross the blood brain barrier after intravenous delivery has opened new opportunities for non-invasive delivery to the brain. However, the efficacy of those vectors is often conditional to the use of a self-complementary AAV genome, which greatly limits the cloning capacity in the genetic backbone and restricts further applications to a limited number of therapeutic genes. Using an *in silico* reconstruction of the viral evolutionary lineage, we recently generated a novel AAV, Anc80, the predicted ancestor of AAV serotypes 1, 2, 8 and 9. Because our initial in vivo evaluation of Anc80 had demonstrated great transduction capabilities, outperforming other conventional AAV such as AAV8, we hypothesize that Anc80 may also emerge as an alternative tool to transduce the neural tissue. To test this hypothesis, we initially compared the capacity of single stranded (ss) Anc80 or ssAAV9 (AAV9 being the most established serotype to cross the blood brain barrier) encoding a Firefly luciferase reporter gene to target the brain ($2.5x10^{12}$vg/kg) after intravenous injection into BALB/c mice. Using noninvasive whole body bioluminescence imaging we observed that ssAnc80 consistently led to higher luciferase signal in the head region than AAV9, a sustained effect over 40 days. To further assess the transduction capacity of Anc80 in the CNS at a cellular level, tail vein injections of $4x10^{13}$vg/kg of ssAnc80, ssAAV9 and scAAV9 encoding for green fluorescent protein (GFP) were performed in C57BL/6 mice. The overall GFP signal intensity could be detected across the entire neural tissue one month after ssAnc80 injection, and was dramatically increased when compared with ssAAV9. Co-staining for GFP and markers of different neural cell types revealed that ssAnc80 mostly transduced neurons and astrocytes, as did ssAAV9 and scAAV9. Using an unbiased stereological approach, we reported that a single injection of ssAnc80 led to the transduction of 13±3.5% of neurons and 29±7.4% of astrocytes, which was significantly higher that ssAAV9 (4.6±1.4% and 5.2±1.2%respectively),but did not reach the levels of scAAV9 (24±9.5% and 45±6.2%, respectively). Interestingly, direct intraparenchymal and intracerebroventricular injections of ssAnc80 also led to enhanced GFP signal as opposed to ssAAV9. Overall, these data suggest that Anc80 may be a highly efficient gene transfer vector for the central nervous system, thus opening novel potential therapeutic avenues using this ancestral AAV variant.

224. Non-Visual Cross Modal Plasticity in the Visual Cortex Does Not Hinder Retinal Gene Therapy Intervention to Improve Sight in Low Vision Patients

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Introduction: While it is well established that the visual cortex is recruited for the use of non-visual sensory functions in blind and low vision individuals, the impact of this adaptive plasticity on vision restoration is largely unknown. The goal of this study is to use functional magnetic resonance imaging (fMRI) to explore how cross modal plasticity in the visual cortex may impact sight restoration. This study specifically examines auditory driven cross modal activity within the visual cortex before and after vision restoration through retinal gene therapy (GT) in a population of patients with type 2 Leber's Congenital Amaurosis (LCA2).

Methods: 8 LCA2 patients and 8 demographically matched normal-sighted controls participated in this study. In a Phase I clinical trial subjects received GT to their worst-seeing eye. In a follow-on (FO) clinical trial, GT was administered to the contralateral eye. Subjects underwent auditory and resting state fMRI before and 3 years after FO GT. Data from fMRI visual task was also analyzed 3 years after GT. Data analysis included group general linear modal analysis using BrainVoyagerQX software. Pearson correlations between the magnitude of visual cortex activations due to audition and vision were also performed.

Results: Auditory fMRI task results revealed significant cross modal visual cortex activity before and after FO visual restoration, with enhanced cross modal activations in the visual cortex 3 years after GT. Normal-sighted controls showed no visual cortex activation while performing the auditory task. Results from visual task stimulation of the left and right eyes 3 years after GT showed significant and widespread occipital lobe activations demonstrating a strong presence for vision. Pearson correlations at the 3-year time point showed trend level and highly significant relationships between visual- versus auditory-evoked activations in the visual cortex for the left and right hemispheres. Resting state fMRI group results showed an increase in functional connectivity between primary auditory cortex and primary visual cortex at the post-GT time point. Functional connectivity between these areas was higher for the right hemisphere at both time points.

Conclusions: Our preliminary results showed a strong presence of cross modal auditory activations and visual activations within the primary visual cortex 3 years after successful vision restoration in a group of LCA2 patients. Based on these results we hypothesize that formation of auditory cross modal function within the primary visual cortex does not hinder retinal intervention to reinstate sight. In fact, we believe that the rise in compensatory cross modal activations following

GT could be due to the strengthening of the visual pathways as a result of GT. This hypothesis is supported by Pearson correlations and resting state fMRI data, which show preferential cross modal strengthening of the right visual pathway that corresponds with Phase I (7/8 patients received superior temporal injection of the right eye) and FO (7/8 patients received central injection to the left eye) GT administration details. Our results suggest that vision restoration through retinal GT allows the visual cortex of low vision patients to respond to both visual and auditory stimuli and that these functions could coexist within the primary visual cortex. Therefore, the formation of the non-visual cross modal functions in the occipital cortex is not an irreversible function to limit visual restoration techniques such as retinal GT.

225. Adeno-Associated Viral Gene Therapy Using PHP.B:NPC1 Ameliorates Disease Phenotype in Mouse Model of Niemann-Pick C1 Disease

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Accessing the central nervous system (CNS) continues to present a challenge when developing therapies for the treatment of neurological diseases. In particular, overcoming the barrier of gene transfer to the brains of animals and patients from the 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, that can transduce a greater number of astrocytes and neurons than AAV9 in the adult mouse CNS. Here we report that a therapeutic AAV-PHP.B vector, systemically delivered, outperforms the naturally occurring serotype, AAV9, in the treatment of a murine model of the rare lysosomal storage disorder Niemann-Pick C1 (NPC1) disease. Approximately 95% of patients have a mutation in *NPC1* which results in either the absence or a significant reduction in functional NPC1, a transmembrane protein in the lysosomal limiting membrane involved in cholesterol transport. Pathology of NPC1 involves lysosomal accumulation of unesterified cholesterol and other associated lipids. Patients typically present with a wide array of neurological symptoms, along with visceral complications including hepatosplenomegaly. Disease progression in the null mouse model of NPC is characterized by weight loss, ataxia, and early death. We previously reported that systemic treatment in *Npc1*$^{-/-}$ mice with AAV9 gene therapy expressing the human *NPC1* gene under the transcriptional control of a ubiquitous promoter (EF1a) improves lifespan and ameliorates disease phenotype. Using a similar study design, we find that an otherwise identical AAV-PHP.B vector improved the lifespan and delayed weight loss in *Npc1*$^{-/-}$ mice more effectively than an AAV9 vector. In addition, preliminary data from AAV-PHP.B-treated *Npc1*$^{-/-}$ mice suggest they perform better than AAV9-treated mice on a balance beam assay and a composite phenotype scoring system, both of which monitor the motor decline and disease progression associated with murine NPC1 disease. These results are the first to show that AAV-PHP.B, a novel serotype engineered for enhanced CNS tropism, improves disease phenotype in an animal model of neurologic disease.

226. Sandhoff (*Hexb*$^{-/-}$) Mice Mount an Immune Response Towards the Novel Human Variant HexM Protein When Treated with scAAV9-*HEXM*

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Sandhoff disease (SD) is a neurodegenerative disorder caused by the toxic accumulation of GM_2 ganglioside in the brain. The β-hexosaminidase A enzyme (HexA), a heterodimer consisting of an α and β subunit, is involved in the catabolism of GM_2 ganglioside. SD is caused by a defective β-subunit, which in turn causes a HexA enzyme deficiency. Recently published, construction of an analogous isoenzyme to HexA, known as HexM, can catabolize GM_2 gangliosides efficiently, and its gene, *HEXM*, fits the cargo capacity of self-complementary adeno-associated virus 9 (scAAV9). A previous study, using the AAV serotype 9 (AAV9) vector expressing HexM showed successful long-term correction of SD in the murine model. However, the use of scAAV9-*HEXM* has the potential to produce an immune response, which can decrease the clinical outcome in treatment for this disease. Using a Sandhoff disease mouse model (*hexb*$^{-/-}$), the immune response against scAAV9-*HEXM* was tested. Adult knockout and heterozygous mice were injected with scAAV9-*HEXM*, purified HexM protein, or vehicle injections. Blood collections were performed at weeks 5, 8, 12, and 15, and mouse endpoints were at 3 weeks or 9 weeks post-injection. The mouse sera obtained from blood collections were tested using enzyme-linked immunosorbent assays (ELISA) to confirm the presence of antibodies against HexM. The splenocytes were isolated to test for IFN-γ cytokine production using the enzyme-linked immunospot (ELISPOT) assay. Knockout and heterozygous treatment groups, injected with scAAV9-*HEXM* or with HexM purified protein and adjuvant, showed high levels of antibody production as compared to the antibody levels in serum of vehicle injected mice. Based on the ELISPOT assay, a high IFN-γ cytokine production was observed 3 weeks after the scAAV9-*HEXM* gene therapy treatment. Similarly, an antibody and T-cell immune responses to viral capsid were also identified. This study demonstrates for the first time that the scAAV9-HexM treatment of Sandhoff disease produces elevated immune response in both knockout and heterozygous Sandhoff mice. It is interesting to note that unpublished data shows long-term correction of Sandhoff disease using the same vector treatment in adult mice. In

order to improve the treatment benefits of gene therapy, experiments are ongoing to investigate the use of immunosuppression to counteract such an immune response and whether that will improve the survival further.

227. *In Vivo* Genome Editing in the G93A-SOD1 Mouse Model of ALS Delays Disease Onset, Improves Motor Function and Extends Survival

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Amyotrophic lateral sclerosis (ALS) is an adult-onset neurological disorder involving the loss of motor neurons in the spinal cord, brainstem, and motor cortex. ALS leads to progressive muscle weakness and atrophy throughout the body, ultimately leading to paralysis and death within 3-5 years of symptom onset. Gain-of-function mutations in the Cu/Zn superoxide dismutase 1 (SOD1) gene account for ~20% of familial forms of the disease and ~2% of all cases. While the mechanism behind SOD1 toxicity is not completely understood, transgenic animals that express the G93A mutant of the human SOD1 protein develop a neurodegenerative disease that emulates the hallmarks of ALS, including motor neuron degeneration, muscle wasting, and paralysis. Genome editing offers an approach to treat autosomal dominant disorders, including many familial forms of ALS, via the disruption of mutant gene function. The RNA-guided Cas9 endonuclease from CRISPR-Cas systems, in particular, has emerged as a versatile genome editing tool. We used the Cas9 nuclease from *Staphylococcus aureus* (SaCas9) to disrupt mutant SOD1 gene expression in the G93A-SOD1 mouse model of ALS following adeno-associated virus-mediated delivery to the spinal cord. CRISPR-Cas9-mediated genome editing resulted in delayed disease onset, improved motor function, reduced muscle atrophy, and extended survival. Additionally, compared to control animals, mice treated by genome editing had, on average, ~50% more spinal cord motor neurons at end-stage, indicating that Cas9 conferred protection to individual motor neurons. These results demonstrate that *in vivo* genome editing has the capability to treat SOD1-linked forms of familial ALS and other central nervous system disorders caused by autosomal dominant mutations.

228. Gene Therapy for a Mouse Model of Glucose Transporter-1 Deficiency Syndrome

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Background: Glucose transporter 1 deficiency syndrome (GLUT1DS, OMIM #606777) is an autosomal dominant disorder caused by haplo-insufficiency of *SLC2A1*, the gene encoding GLUT1. Heterozygous mutation of *SLC2A1* results in impaired hexose transport into the brain and finally irreversible neurologic disorders. Part of seizures can be treated with ketogenic diet but movement disorder and intellectual disability persist. We generated the tyrosine-mutant AAV9/3 vector in which *SLC2A1-myc-DDK* was expressed under the neuron-specific synapsin I promoter (AAV-h*SLC2A1*). We also examined if AAV-h*SLC2A1* administration can lead to functional improvement in a mouse model of GLUT1DS; heterozygous knock-out of the *Glut1* gene ($GLUT1^{+/-}$) that mimics the human GLUT1DS. **Methods:** AAV-h*SLC2A1* was injected into the peritoneum (systemic; 1.85×10^{11} vg per mouse) or bilateral lateral ventricles (local; 1.85×10^{10} vg per mouse) of $GLUT1^{+/-}$ mice. Mice were analyzed for mRNA and protein expression of GLUT1 in brain tissue, motor function using rota-rod test and footprint test, and blood and cerebrospinal fluid (CSF) glucose levels. Additionally, we generated untagged AAV-h*SLC2A1* to assume clinical application and analyzed in the same way. **Results:** In quantitative PCR, total amount of mRNA of *GLUT1* was increased 2.4-fold in intra-cerebroventricular injection group ($P<0.05$), and 1.6-fold in intraperitoneal injection group ($P=0.09$) than $GLUT1^{+/-}$ mice. Exogenous GLUT1 was expressed mainly in neurons, and partially in endothelial cells and oligodendrocytes. After intra-cerebroventricular injection of untagged AAV-h*SLC2A1*, GLUT1-immunoreactive cells were confirmed in the cerebral cortex and hippocampus nearby injection site. The motor function test and CSF/blood glucose ratio were significantly improved following intra-cerebroventricular injection. **Conclusion:** Administration of AAV-h*SLC2A1* to $GLUT1^{+/-}$ mice produced exogenous GLUT1 expression in neural cells, improved CSF glucose level, and motor function. Gene therapy using the AAV-h*SLC2A1* vector is a potential treatment for human GLUT1DS patients. In addition, we are investigating other promoters that approximate human physiological GLUT1 expression.

229. Disruption of Water Homeostasis Affects AAV Transport Across the Blood-Brain Barrier

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In the brain, the water transport channel Aquaporin 4 (*Aqp4*) is known to be a major driver of bulk fluid flow. This 'glymphatic' system has been identified as a macroscopic clearance mechanism for removal of interstitial solutes from the CNS. Reduced glymphatic water transport and diminished clearance of parenchymal solutes is a hallmark feature of aging and neurological disease. Our lab recently demonstrated that deregulation of glymphatic transport in AQP4-/- as well as aged brains markedly affects the parenchymal spread, clearance, and gene transfer efficiency of AAV vectors following intra-CSF injection. Here, we evaluate the impact of *Aqp4* deletion on AAV transport across the blood-brain barrier (BBB). We first observed increased vascular uptake of intravenously (i.v.) administered horse radish peroxidase (HRP) in $AQP4^{-/-}$ mice compared to B6/129 controls. Pretreatment with low dose histamine resulted in penetration of i.v. administered HRP into the brain parenchyma in AQP4-/- mice. These results implied that, although not compromised, vascular/capillary function might be affected in AQP4-/- mice. Strikingly, i.v. administration of AAV1, which does not traverse the vasculature, resulted in significantly increased transduction of brain endothelial cells. Importantly, the number of transduced cortical astroglia was over an order of magnitude higher in AQP4-/- mice compared to control supporting increased AAV1 uptake into perivascular astroglial endfeet from the endothelium. In contrast, AAV9 and AAVrh.10, which are known to cross the BBB, showed no changes in their ability to transduce the brain following i.v. administration. Thus, AAV1 and AAV9/rh.10 appear to interact with the vasculature in a distinct fashion and exploit distinct mechanisms for endothelial transport. Further, since *Aqp4* dysfunction has been noted by our group and others in aging mice, observations pertaining to AAV transport across the BBB in mouse models of aging and disease will also be presented. In summary, our findings highlight the role of *Aqp4* in maintaining BBB homeostasis, which in turn affects viral transduction in the brain. The study has clinical implications that could inform CNS gene therapy trials, but also constitutes a fundamental step towards understanding the mechanisms by which certain AAV serotypes cross the BBB.

230. Towards Intrastromal rAAV *PAX6* Gene Therapy for the Congenital Blindness Disorder Aniridia

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Aniridia is a rare congenital disorder of the eye caused by mutations in the gene Paired Box 6 (*PAX6*). *PAX6* is an important transcription factor for the formation of the eye; harbouring a single loss-of-function *PAX6* mutation disrupts ocular development, leading to congenital retinal, lens, iris, and corneal malformations. These malformations result in low vision from birth, which continues to deteriorate in early life, often leading to blindness in young adulthood. The progressive nature of aniridia opens a window in which vision saving therapeutics could be administered. A major contributor to progressive vision loss in aniridia is the degeneration of the cornea, which can become opaque and vascularized in the first decades of life. Therefore, the cornea is an important target tissue for new vision-saving treatments. Here, using recombinant adeno-associated viruses (rAAVs), *Xenopus laevis*, and the small eye (*Sey*) mouse model of aniridia, we lay the foundation for developing new gene therapy treatments for aniridia.

To distinguish between endogenously expressed and rAAV delivered PAX6, a 3xFLAG-tag was added to either the N- or C-terminal of PAX6 (3xFLAG/PAX6 and PAX6/3xFLAG respectively). Protein function was assessed by injection of *in vitro* transcribed 3xFLAG/PAX6, PAX6/3xFLAG, *PAX6*, and EmGFP mRNA into *X. laevis* embryos. The resulting tadpoles were fixed and evaluated by light microscopy 14 days after injection, revealing that 3xFLAG/PAX6 and PAX6 induce the formation of ectopic eye structures at a similar rate. Consequently, 3xFLAG/PAX6 was selected for use in rAAV.

3xFLAG/PAX6 and EmGFP open reading frames were cloned into a custom "plug-and-play" single stranded rAAV genome. The genomes were packaged into rAAV serotype 9 (rAAV9) at the University of Pennsylvania vector core at a minimum titer of $1x10^{13}$ vg/mL. Red fluorescent microbeads (FluoSpheres, Fisher Cat. F8793), which served as an injection tracer, were mixed with rAAV9 and administered directly into the mouse cornea by intrastromal injection. Histological examination of virus & FluoSphere-injected mouse corneas revealed that the FluoSpheres were retained within the stroma, without leakage into the neighbouring corneal layers or ocular structures. EmGFP expression was detected as early as three-days after virus administration by both epifluorescence and immunofluorescent staining. Expression was successfully achieved in both the central and peripheral cornea, spanning all three major corneal layers: the epithelium, stroma, and endothelium.

In conclusion, intrastromal administration of rAAV9 can successfully transduce all three layers of the mouse cornea, an important target tissue for treating aniridia. Robust transduction of these tissues lays the foundation for preclinical trials of a *PAX6*-gene therapy for aniridia.

231. Spermine Accumulation Drives Abnormal Neurite Development in Hurler Syndrome That Is Reversed by Gene Therapy

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The mucopolysaccharidoses (MPSs) are a family of genetic disorders caused by deficiency of enzymes required for lysosomal degradation of glycosaminoglycans (GAGs), leading to accumulation of GAGs in tissues and debilitating somatic disease. While there are clear phenotypic similarities among MPSs, there are striking differences in CNS manifestations; in MPS types IH (Hurler syndrome), II, III and VII, patients generally exhibit a marked decline in cognitive development at 1-2 years of age. In contrast, MPS types IV and VI show no cognitive involvement, despite similar systemic signs of GAG storage. This difference in phenotype correlates with heparan sulfate (HS) storage; all MPS types that involve accumulation of HS exhibit cognitive dysfunction, whereas MPS IV and VI, which are instead characterized by defects in keratan and dermatan sulfate catabolism, do not. A mechanistic link between HS accumulation and cognitive impairment has not been established. Gangliosides accumulate in neurons of MPS IH patients and are hypothesized to contribute to the neurological phenotype, given that disorders of ganglioside catabolism (e.g., Tay-Sachs and Sandhoff disease) have severe neurological manifestations. However, ganglioside accumulation in neurons occurs to a similar degree in MPS VI, a disease without cognitive impairment, indicating that elevated brain gangliosides are unlikely to be directly related to CNS pathogenesis in MPS IH, II, III, and VII.

In the current study, we conducted high-throughput LC/MS and GC/MS global metabolite profiling of CSF from 15 MPS I and 15 wild-type canines, and identified a marked elevation in the concentration of the polyamine spermine. Spermine is transported in a heparan sulfate-dependent manner, and has been associated with promoting neurite outgrowth after axon injury. Next, using a targeted LC/MS assay to specifically detect spermine, we identified up to a 30-fold elevation in the CSF of MPS IH patients. To determine the role of spermine in cognitive impairment in MPS I, we cultured isolated E18 murine cortical neurons. We found that *in vitro* neurite overgrowth by MPS I neurons was spermine-dependent, and treatment with exogenous spermine induced excess neurite growth in wild-type neurons. Finally, we evaluated the impact of CNS-directed gene therapy on spermine levels in MPS I canines. Treated MPS I canines exhibited reduced CSF spermine levels and normalized cortical expression of GAP43, a key protein involved in neurite outgrowth. These findings implicate defects in polyamine metabolism in the pathogenesis of the CNS manifestations of MPSs, and suggest that CSF spermine may be an informative biomarker for evaluating the efficacy of novel therapeutics.

232. HSV Vectors Retargeted for Transgene Delivery Restricted to NGF Receptor-Bearing, Pain-Sensing C-fibers

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Chronic pain represents a major cause of morbidity and effective pain therapy remains a significant unmet medical need. The standard of care relies primarily on systemic drug therapies that do not target the site of pain sensation. These therapies often have limited effectiveness, deleterious side effects, and induce tolerance. Herpes simplex virus (HSV)-based gene therapy vectors can deliver therapeutic genes to sensory nerve afferents where pain is arising, representing an attractive alternative to drug therapy. Our goal is to develop a transductionally retargeted HSV vector to selectively deliver therapeutic genes to only those neurons that are activated in chronic pain states. NGF/receptor (TrkA) signaling mediates the pain response associated with inflammatory hyperalgesia and neuropathic pain conditions, making TrkA-expressing cells an important target for chronic pain gene therapy. To obtain a fully retargeted HSV, the viral envelope attachment/entry component glycoprotein D (gD) can be modified to eliminate recognition of its canonical receptors (HVEM and nectin1) and incorporate a cognate ligand for virus entry through a novel receptor. Accordingly, we replaced the N-terminal signal peptide and HVEM binding domain of gD with pre-pro-(pp)NGF to create a TrkA-targeting protein, gD:ppNGF(Y38), that can still bind nectin1. Virus expressing gD:ppNGF(Y38) was propagated on cells expressing nectin1 and purified virus was shown to enter gD receptor-deficient J1.1-2 cells only following transduction of the cells with TrkA (J/TrkA cells). To improve vector propagation on these cells, we used genetic selection to isolate retargeted virus variants that can enter and spread with increased efficiency on J/TrkA cells. Characterization of one effective variant, J4H, revealed the presence of mutations in viral genes encoding envelope glycoproteins involved in virus-cell fusion (gH) and virus cell-to-cell spread (gE and gI). Introduction of the gH mutation into the parental virus resulted in enhanced entry but without significant improvement of virus spread. We are also exploring the phenotypes of the gE and gI mutations separately and together. In addition, we have created a J4H derivative, J4HΔ38, that irreversibly eliminates nectin1 binding but preserves enhanced entry and spread on J/TrkA cells. Preliminary results indicate that J4HΔ38 specifically and efficiently infects TrkA-expressing primary sensory neurons in culture, and *in vivo* infections are planned. We suggest that HSV retargeting to TrkA will provide a means for delivery of therapeutic gene products exclusively to those neurons that are at the root of pain sensation.

233. Codon Optimization of the Menkes Gene Greatly Enhances ATP7A Expression

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Menkes disease is an X-linked recessive neurometabolic disorder with high morbidity and mortality caused by a defective transmembrane copper transporter, ATP7A. Our previous results demonstrated successful rescue of the Menkes disease mouse model using cerebrospinal fluid (CSF)-directed AAV9 ($1.6x10^{10}$vg) gene therapy. We utilized a reduced size (rs) version of human ATP7A (3.3kb, truncated from the 4.5kb normal size) due to the packaging limitations of AAV. We demonstrated that the rsATP7A protein pumps copper efficiently (approximately 85% of normal) and traffics properly between the trans-Golgi compartment and plasma membrane.

We recently generated a novel cDNA sequence of rsATP7A, based on codon usage bias. We inserted this codon-optimized (co) version in pTR-CAG expression plasmid and assessed recombinant protein expression after transfecting HEK293T cells. Expression of co-rsATP7A was 40 times higher than rsATP7A based on careful densitometric analyses. Preliminary experimental therapeutic results in the Menkes mouse model suggest successful rescue can be achieved at a lower dose of AAV9 ($1.6x10^{9}$vg) when the co-rsATP7A construct is used (in combination with subcutaneous Copper Histidinate). Taken together, our results provide additional rationale in support of CSF-directed, AAV9-mediated gene addition for treatment of Menkes disease.

234. Targeting TDP-43 in Mitochondria to Treat Neurodegenerative Diseases

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Dominant missense mutations in TAR DNA-binding protein 43 (TDP-43) cause amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), and the cytoplasmic accumulation of TDP-43 represents a pathological hallmark in ALS, FTD and Alzheimer's disease (AD). We have found that accumulated cytoplasmic TDP-43 in degenerating neurons of patients with ALS, FTD or AD mainly resides inside of mitochondria. Within mitochondria, TDP-43 preferentially binds mitochondria-transcribed messenger RNAs (mRNAs) encoding respiratory complex I subunit ND3 and ND6, impairs their expression and specifically causes complex I disassembly. Based on identified motifs critical for TDP-43 mitochondrial localization, we have synthesized a competitive inhibitory peptide that can prevent the accumulation of TDP-43 in mitochondria and abolish TDP-43-induced mitochondrial dysfunction and neuronal loss. Excitingly, suppression of TDP-43 mitochondrial localization by this synthetic inhibitory peptide is sufficient to prevent and even reverse ALS or FTD-related phenotypes in two TDP-43 transgenic mouse models before or after symptom onset. Thus, our study suggests mitochondrial TDP-43 as a promising novel therapeutic target for TDP-43-linked neurodegenerative diseases.

235. Efficient Transduction of Inner Retina by Surgical Internal Limiting Membrane (ILM) Peeling Before Intravitreal AAV Vector Injection in Cynomolgus Monkeys

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[Objective] The adeno-associated virus (AAV) vector is an ideal tool for retinal gene therapy. There are two administration routes for retinal gene transduction, subretinal injection (SR) and intravitreal injection (IV). SR is used in many studies because of its efficiency of gene transduction of outer retina. However, SR induces an iatrogenic retinal detachment, which causes several adverse effects. On the other hand, IV is considered to be safer than SR, although the transduction efficiency in large animals was insufficient. Some reports suggest that vitreous and internal limiting membrane (ILM) react as the barriers to gene transduction and resulted in low transduction efficiency in large animals. To overcome this issue, we performed vitrectomy (VIT) and ILM peeling before AAV vector injection. [Methods] We used three female cynomolgus monkeys (aged 10-13 years old). Among the six eyes, two eyes received standard 3-port VIT (VIT group), two eyes received ILM peeling in addition to the standard 3-port VIT (VIT+ILM group), and the remaining two received no pretreatment (IV group) at 4 weeks before AAV injection. All eyes were penetrated by 30-G needle into the vitreous at the pars plana, and 50 μl of triple-mutated self-complementary AAV serotype 2 vector (1.9×10^{13} v.g./ml) expressing enhanced green fluorescent protein (GFP) was administered. Nineteen weeks after intravitreal injection of AAV vector, transduction efficiency was analyzed. To detect adverse effects, the retinas were monitored using color fundus photography, optical coherence tomography (OCT) and electroretinography (ERG). [Results] Strong GFP expression was detected in the peeled ILM area in the VIT+ILM group, whereas little expression of GFP was detected in the IV and VIT groups. Intraocular inflammation was observed in 4 of 6 eyes. This inflammation was transient and it was cured without any treatment. No morphological changes were detected on color fundus images or OCT. Transient reduction of ERG amplitude was detected during the period of intraocular inflammation. [Conclusions] These results indicate that surgical ILM peeling before intravitreal AAV vector administration would be safe and useful for efficient transduction of the nonhuman primate retina. This transduction strategy would provide therapeutic benefits for the treatment of retinal diseases.

236. Characterisation of a Tissue Specific Retinal Promoter

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The advent of capsid modified adeno-associated virus (AAV) serotypes has enabled scientists to target an increasingly diverse number of cell targets, particularly in the mammalian retina, with greater potency. It is therefore timely to reinvigorate efforts to determine efficient tissue specific promoters to restrict gene expression to the target cell of interest. In this study, we utilise *in silico* gene analysis to identify a proximal promoter to mediate gene expression in retinal ganglion cells (RGC) and demonstrate its efficacy *in vivo*, following adeno-associated virus (AAV) delivery to the mouse retina.

We analysed the upstream region of genes with expression profiles limited to the ganglion cell layer (GCL) for sequence conservation across placental mammals, weighted by enriched expression within the GCL. Conservation of upstream sequence was used as a proxy for putative promoter function. Furthermore, we restricted the region analysed to the 2.5kb directly upstream of the transcriptional start site in order to maximise application of the promoter in AAV. The lead proximal promoter element, termed GCP1, was cloned upstream of an enhanced green fluorescence protein gene (EGFP) and AAV2 generated. AAV2.GCP1-EGFP was evaluated in wild type mice following intravitreal injection in comparison to AAV2.CMV.EGFP, a strong universal viral promoter. Significantly, AAV2.GCP1-EGFP expression was restricted to the GCL, while CMV-EGFP expression was seen throughout the GCL, inner nuclear layer (INL) and outer nuclear layer (ONL). To further delineate the specificity of GCP1 for RGCs, immunohistochemistry was performed for GFP, brain-specific homeobox/POU domain protein 3A (Brn3a), choline acetyltransferase (ChAT) and gamma-aminobutyric acid (GABA). AAV2.GCP1-EGFP demonstrated a selective tropism for RGCs, compared to AAV2.CMV-EGFP which expressed in a greater number of amacrine cells.

We conclude that GCP1 represents a novel 2.2kb promoter that mediates robust gene expression in RGCs when administered using an AAV2 vector. Importantly, the data suggest that GCP1 may have potential for use in therapies targeted towards RGCs without compromising the magnitude of promoter-mediated expression. Furthermore, GCP1 demonstrates effectively that sequence conservation in concert with gene expression profiles can be used to infer putative promoter sequences.

237. Expression of a Sodium Channel Subunit in Inhibitory Interneurons Using Adeno-Associated Viral Vectors

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GABAergic inhibitory interneurons are crucial for maintaining the balance between excitatory and inhibitory neuronal networks. Functional impairment of inhibitory interneurons causes febrile seizures, epilepsy, and mental disorders. Dravet syndrome is a genetic disorder caused by mutations in voltage-gated sodium channels that mediate action potentials in neurons. Some studies indicate that sodium channels expressed in interneurons are particularly affected in Dravet Syndrome. There are no effective pharmacological therapies for Dravet syndrome. We generated an AAV9 encoding a chaperone of the mouse sodium channel alpha subunit - a sodium channel beta subunit (Navβ), driven by a mouse *Gad* promoter for expression in inhibitory interneurons. The AAV was injected into the C57BL/6 mouse brain by intracerebroventricular injection. Viral Navβ expression was analyzed at postnatal day 56. The virally expressed Navβ transgene was distributed mainly in neurons of the frontal cortex and hippocampus. Ongoing experiments will assess the efficacy of this vector in ameliorating epileptic seizures and extending life span in a mouse model of Dravet Syndrome. The work is supported by the Canadian Institutes for Health Research and Dravet.ca.

238. Disease Modeling in Patient-Derived Cells Harboring Mutations in *ABCA4*

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Mutations in the large polymorphic gene *ABCA4* contribute to a variety of inherited retinal phenotypes ranging from Stargardt disease to a severe condition resembling retinitis pigmentosa (RP). Collectively, these mutations represent the most common cause of autosomal recessive retinal dystrophies. The observation of several clinical phenotypes signifies a relationship between the contribution of each allele and disease severity. The development of induced pluripotent stem cell (iPSC) technologies and the recent genome editing technology CRISPR-Cas9 provide an opportunity to investigate how alleles contribute to disease progression *in vitro* in patients' own cells. Moreover, using patient-derived cells is important for the design and execution of clinical trials testing gene and cell therapies. To that end, we classified patients carrying mutations in *ABCA4* into three separate categories; 1) mild, 2) moderate, 3) and severe, based on clinical phenotype. We reprogrammed patient fibroblasts from seven individuals representing each of the three classes via Sendai viruses expressing the Yamanaka factors *OCT4, SOX2, KLF4,* and *cMYC.* RT-PCR and Sanger sequencing analysis in iPSCs from two patients carrying a mild splice mutation allele (V2114V c.6342 G>A) revealed a 47bp deletion in exon 46. To correct the mutation, we designed single guide RNAs and cloned them into a dual-expression plasmid carrying the humanized Cas9 cDNA from *S. pyogenes*. To screen for efficiency, we transfected these plasmids into HEK293T cells. T7E1 nuclease assays and Sanger sequencing analysis indicated successful modification at the target locus. We selected the guide with the highest efficiency and designed a donor template carrying ~500 bp of homologous sequence flanking the repair sequence in addition to a floxed puromycin selection cassette. To test homology-directed repair, we transfected the guide and donor plasmids into HEK293T cells and selected under puromycin pressure for one week. PCR analysis demonstrated successful repair in cells treated with both the guide and donor plasmids compared to untreated controls. These reagents will be used in patient-derived cells to study the degree to which gene correction ameliorates disease progression *in vitro.*

239. Investigating miRNA Populations of Similar Neuronal Types Projecting to Different Nuclei

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Expression of micro RNAs (miRNA) can potentially tell us a lot about the state of specific cells. Over- or underexpression of a specific type of miRNA may be a sign, or a cause of dysregulation of normal cell function, which when occurring in sufficiently large numbers of cells may lead to disease. Differences in miRNA population may also help determine cell type. These facts can be exploited both to detect potential changes in cell states, but also to regulate expression of transgenes [1].

However, this first requires knowledge of which miRNAs are expressed in which cells. We were interested in looking at the miRNA profiles of similar neurons that project to different nuclei. To sequence the miRNA profiles of these neurons we decided to use microRNA immunoprecipitation (miRIP). MiRIP uses the fact that miRNA naturally binds to a protein called Argonaute 2 (Ago2) in the cell. By expressing a fusion protein of Ago2 and GFP in specific neurons and through the use of affinity chromatography with antibodies against GFP, we could extract the miRNA of the cells expressing the fusion protein [2].

As our first target we decided to look at the direct and indirect pathway of the basal ganglia. These two pathways consist of similar neurons (Medium spiny neurons), that project to either the substantia nigra pars compacta or the globus pallidus. We therefore injected a vector with a high degree of retrograde transport into either the globus pallidus or substantia nigra, expressing a GFP-Ago2 fusion protein. Initial tests with this AAV using a modified AAV2 capsid protein showed promising results with large amounts of cell bodies in the striatum transduced. The striatum was then collected in order to perform miRIP on the transduced neurons.

Novel Models for Genetic, Metabolic and Endocrine Disorders

240. The First Viable Mouse Model of *cblC* Type Combined Methylmalonic Acidemia and Hyperhomocysteinemia: AAV Gene Therapy Rescues Neonatal Lethality

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Combined methylmalonic acidemia and homocysteinemia, *cblC* type (*cblC*), is the most common inborn error of cobalamin metabolism and is caused by mutations in the *MMACHC* gene. MMACHC transports and processes intracellular cobalamin (vitamin B12) into its two active cofactors, 5'-adenosylcobalamin and methylcobalamin, necessary for the enzymatic reactions of methylmalonyl-CoA mutase and methionine synthase, respectively. Mutations in *MMACHC* result in methylmalonic acidemia, hyperhomocysteinemia and hypomethioninemia. Disease manifestations include growth failure, anemia, heart defects, developmental delay, neurocognitive impairment and a progressive maculopathy and pigmentary retinopathy that causes blindness, usually by the end of the first decade. Treatment with cobalamin and other supplements improves the metabolic abnormalities but fails to improve some manifestations of *cblC,* in particular the eye disease. To explore disease pathophysiology and develop AAV gene therapy for *cblC* deficiency, we first created a viable mouse model using TALENs to edit the murine *Mmachc* gene, near the location of the most common mutation in humans, c.271dupA. Two alleles were investigated: c.165_166delAC p.P56CfsX4 (Δ2) and c.162_164delCAC p.S54_T55delinsR (Δ3). $Mmachc^{\Delta2/\Delta2}$ and $Mmachc^{\Delta3/\Delta3}$ homozygous mutant mice displayed reduced survival, severe growth retardation, and massive metabolic perturbations. We observed disturbed Mendelian ratios with a decrease in number of mutants at birth ($p<0.05$ for both Δ2 and Δ3). The median survival was less than 7 days with 90% of the mutant mice perishing before 3 weeks (Δ2 n=13; Δ3 n=42 $p<0.0001$). The weights of $Mmachc^{\Delta3/\Delta3}$ mice were reduced relative to heterozygote and wild type littermates (n=15, $p<0.0001$). $Mmachc^{\Delta2/\Delta2}$ and $Mmachc^{\Delta3/\Delta3}$ mice (n=4, n=6) recapitulated the biochemical features of *cblC*, with significantly elevated plasma methylmalonic acid, homocysteine, cystathionine and decreased methionine compared to littermate controls (n=7) ($p<0.05$ for all metabolites). To assess the potential for gene therapy as a treatment for *cblC*, we generated two AAV vectors, rAAVrh10-CBA-m*Mmachc* and rAAV9-CBA-h*MMACHC,* that were delivered via a single intrahepatic injection in the neonatal period (1×10^{11} GC). AAV treatment was compared to treatment with weekly injections of hydroxocobalamin, the standard therapy. $Mmachc^{\Delta3/\Delta3}$ mice treated with AAV vectors (AAVrh10 n=11, AAV9 n=5) displayed dramatically improved clinical appearance with improved growth ($p= 0.0568$), and increased survival ($p<0.0001$ for both vectors), with the oldest treated mutants currently living beyond one year, similar to the standard hydroxocobalamin therapy. In mutant mice treated with rAAVrh10-CBA-m*Mmachc,* mean plasma methylmalonic acid was 160uM at 10 months of age (n=3), approximately three-fold lower than untreated mice at 1 month of age (mean, 457uM, n=4). The effect of combined rAAV9-CBA-h*MMACHC* and hydroxocobalamin therapy on weight, survival, and clinical outcome is currently being assessed. Our results demonstrate that AAV gene delivery of *MMACHC* represents a new therapy for *cblC* which can treat the systemic manifestations of this relatively common and devastating inborn error of metabolism.

241. Novel Transplantation Modalities for Generating Transcriptionally Dependable New Microglia from Hematopoietic Stem and Progenitor Cells

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Recent evidences indicate that hematopoietic stem and progenitor cells (HSPCs) can serve as vehicles for therapeutic molecule delivery to the brain by contributing to the turnover of resident myeloid cell populations. However, the nature of the cells reconstituted after transplantation and whether they could comprise bona fide microglia remain to be assessed. Moreover, the still limited efficiency of the process and its long timing advocates for novel strategies to enhance the potential associated clinical benefit for patients. In this work, we firstly demonstrate that HSPC transplantation can generate transcriptionally-dependable new microglia through a stepwise process reminiscent of physiological post-natal microglia maturation and new forming microglia like cells are transcriptionally distinct from bone marrow resident or circulating myeloid cells. We also unambiguously identified in the long-term hematopoietic stem cell compartment the cell fraction within murine and human HSPCs that mostly retains the ability to reconstitute microglia upon transplantation, likely favored in their trafficking to the brain by CXCR4 expression. Finally, generation of microglia-like cells of donor origin was firstly obtained also upon intra-cerebral ventricular delivery of HSPCs and this novel delivery route is associated to a clinically relevant faster and more widespread microglia replacement compared to systemic injection, confirming our original hypothesis that microgliosis could derive from an independent seeding of the brain by the intra-venously transplanted HSPCs. Overall, this work supports the relevance and feasibility of employing HSPCs for exerting therapeutic effects in the central nervous system (CNS), and identifies novel modalities, based on selection of populations to be transplanted and use of innovative transplant routes, for increasing the actual contribution of the transplanted cells to microgliosis and their therapeutic activity.

242. Long-Term Rescue of a Hypomorphic Lethal Murine Model of Citrullinemia Type I by Liver-Directed, AAV8-Mediated Gene Therapy

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Citrullinemia type I (CTLN1) is an autosomal, recessive disorder of the urea cycle caused by a deficiency of argininosuccinate synthetase 1 (ASS1). The clinical spectrum of CTLN1 ranges from a severe neonatal onset form to a milder form with later onset. Affected patients have persistent elevated plasma citrulline levels and are at risk of life-threatening elevation of ammonia that can lead to irreversible cognitive impairment, coma, and death. Current treatment for CTLN1 patients, which includes a low protein diet, supplementation of arginine and administration of nitrogen scavengers, is often unable to prevent ongoing hyperammonemic crises. Liver transplantation has shown successful reduction of plasma ammonia and citrulline levels, but donor liver is limiting, the procedure itself carries significant morbidity, and immunosuppressive drugs are necessary for the duration of the graft. Therefore, there is a need for other approaches to therapy for CTLN1. AAV vector-based gene therapy would provide an alternative to current treatment options as long as the vector delivers sufficient and sustained transgene expression in the liver without substantial toxicity. We generated several candidate AAV8 vectors for CTLN1 with different liver-specific promoters, introns, and cDNA sequences (native or codon-optimized h*ASS1* cDNA). *In vivo* evaluation of vectors was performed in a murine model of CTLN1 ($ASS1^{fold/fold}$). Homozygous $ASS1^{fold/fold}$ (*fold*) mice carry a hypomorphic mutation and display lethality after weaning. Half of the untreated *fold* mice perished before the age of 12 weeks old, while a few (5%) lived up to 5 months. In addition to significantly elevated plasma citrulline levels, untreated *fold* mice have significantly reduced body weight, variable elevated plasma ammonia levels and urine orotic acid levels, and they are not fertile. Four-week-old *fold* mice were dosed via retro-orbital or intraperitoneal injection with 3x10^11 GC or 1x10^11 GC of vector. Reduction of plasma citrulline levels was chosen as the main criteria to differentiate the performance of different vectors. A lead vector containing the ApoE enhancer-alpha 1 antitrypsin promoter and the beta globulin intervening sequence 2 achieved 77% reduction of citrulline levels two weeks post vector administration at the dose of 1x10^11 GC. Intron may play an important role in expression of ASS1 and vectors carrying the same promoter with other introns, or no intron, showed significantly perturbed efficiency in reducing citrulline levels. Vector with native cDNA sequences performed slightly better than a vector with codon-optimized cDNA sequences. *Fold* mice treated with the top candidate vectors gained weight, became fertile, and survived more than 9 months (still on-going). The various vectors developed in this study have the potential to become clinical candidates for gene therapy of CTLN1.

243. A Mouse Model of *Mmaa* (*cblA*) Deficiency Replicates the Infantile Phenotype of Isolated Methylmalonic Acidemia (MMA)

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Isolated methylmalonic acidemia (MMA) is a group of inherited metabolic disorders characterized by the inability to generate succinyl-CoA from methylmalonyl-CoA as a result of mutations in the methylmalonyl-CoA mutase (MUT) gene or deficiencies in enzymes involved in the synthesis (MMAB or *cblB*) or transfer (MMAA or *cblA*) of its cofactor, 5'-deoxyadenosylcobalamin (Adocbl). The MMAA protein is proposed to function in the gated transfer of Adocbl, as well as the turnover, protection, and rescue of the MUT enzyme.

MMA patients have a variety of presentations when not diagnosed by newborn screening. While those with *cblB* and *mut* subtypes of MMA often present with neonatal crisis, patients with MMAA deficiency have a more variable disease onset, and classically suffer from severe failure to thrive when they transition to solid foods after an uncomplicated, early neonatal course. Basal ganglia strokes, renal tubular disease, optic nerve atrophy and metabolic instability can complicate the disease for those who survive. One unique feature of patients with *cblA* MMA is the universal response to injectable cobalamin, which induces a clinical and partial biochemical response.

We previously generated an *Mmaa* deletion mutant mouse model using homologous recombination and noted variable early lethality and a severe phenotype on the C57Bl6 enriched background, with approximately 50% perishing by one month of age. To create a more facile model that avoids neonatal lethality, yet demonstrates prolonged survival without treatment and the full spectrum of *cblA*-associated disease manifestations, we bred the parental strain to FVBN, and then intercrossed the F1 progeny to make [C57Bl6xFVBN] *Mmaa*$^{-/-}$ mice. These animals more accurately model the human infantile presentation of *cblA* class MMA as they display uniform survival to weaning with preservation of weight. Post-weaning, *Mmaa*$^{-/-}$ mice experience severe growth failure and hypoactivity, recapitulating the failure to thrive and decreased activity seen in untreated cblA patients. Like the patients, the mice show partial responsiveness to hydroxycobalamin. *Mmaa*$^{-/-}$ mice were treated twice a week for two weeks with hydroxycobalamin. A statistically significant weight increase (<0.05) was seen in the treated *Mmaa*$^{-/-}$ males, whereas untreated mutants remained stunted at less than 50% of the control littermate weight, as shown in Figure 1. These mice expand the ability to model MMA without the obligate requirement for a rescue transgene, and should be useful to study pathophysiology, gene and cell therapy, and explore metabolic pathway engineering and synthetic biology approaches to develop global treatments for propionate oxidation disorders.

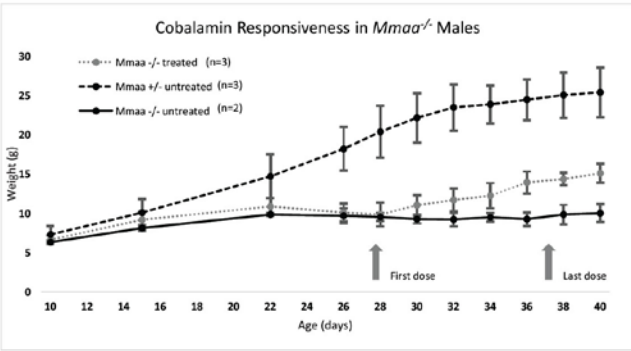

Figure 1: Hydroxycobalamin was injected subcutaneously every 3 days, starting on Day 28. The last dose was given on Day 37.

244. Effect of Aerobic and Resistance Exercise Training on Fat Derived Mesenchymal Stromal Cells (MSCs) in Subjects with Prediabetes

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The effect of aerobic exercise has been studied extensively using various inflammatory biomarkers, however exercise effect on critical cells such as endothelium and fat in order to gauge cellular memory effect post exercise training has not been studied adequately. Previously, we have shown stem or progenitor cells such as endothelial progenitor cells (EPCs, defined as CD34+ cells) can act as a strong cellular biomarker of endothelial function following exercise training as an intervention. In this study, we are examining the effect of aerobic exercise on adipocyte derived MSCs as a cellular surrogate of fat activity and metabolism. **Methods:** In an on-going study, overweight and obese subjects (n=10) with prediabetes (HbA1C 5.7-6.4), were enrolled in a 12 week exercise intervention study. The biweekly exercise sessions were supervised by a trained exercise physiologist and consisted of a 1 hour sessions that included warm-up and cool-down and 30 min of combined aerobic and resistance training at an exercise intensity of 50-80% of heart rate reserve. The patients were also encouraged to be physically active during the rest of the week. Subcutaneous abdominal fat biopsies were obtained and fat derived stromal cells were cultured in vitro for 2-3 weeks. MSCs were analyzed for mRNA gene expression (qRT-PCR) and cellular oxygen consumption rate (OCR), pre and post 12 week exercise. **Results:** Gene expression analysis of the stromal cells pre and post exercise showed that glucose transporter, GLUT1 upregulated significantly (fold increase = 2.5, p= 0.01), with a trend of improvement in certain genes such SOD3, Catalase, NRF1, UCP1 and PRDM16 (fold increase= 2.2, 1.5, 1.5, 3.2 and 1.5, p values= 0.2, 0.25, 0.17, 0.25, and 0.42, respectively), pre vs post exercise. . Though Basal and maximal oxygen consumption rate (OCR) did not change significantly but the ratio of area under the curve of OCR graphs, pre-exercise-MSC/MSC vs post-exercise-MSC/ MSC increased with exercise (pre-exercise/ MSC= .623 and post-exercise/ MSC= 1.11). **Conclusion:** Our study indicates that exercise training improves fat derived MSC cell respiration and gene expression. Exercise training augments cellular glucose transporters (GLUT1), intra and extra-cellular antioxidants (Catalase & SOD3), and possibly increase differentiation of white subcutaneous adipocytes towards brown-like (evidenced by NRF-1 and UCP1 upregulation) cells.

245. Cell Reprogramming for Disease Modelling of Primary Hyperoxaluria Type I

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Disease models of rare metabolic diseases are essential to understand the molecular mechanisms that drive pathogenesis and enable the development of novel therapies. Cell reprogramming offers a valuable tool to develop more relevant and patient-specific disease models. Primary hiperoxaluria type 1 (PH1) is an inherited autosomal rare metabolic disease, caused by deficiencies in the hepatic alanine:glyoxylate aminotransferase, due to mutations in *AGXT* gene. In this work we describe two different strategies to develop *in vitro* PH1models by cell reprogramming. For the first strategy, we used dermal fibroblasts and peripheral blood mononuclear cells homozygous for p.G170R, the most common PH1-associated mutation, and for p.I244T, a mutation highly prevalent in Canary Islands due to a founder effect, to generate patient-specific induced pluripotent stem cells (iPSCs). Those iPSC were differentiated to hepatocyte-like-cells (HLCs) using established protocols mimicking developmental cues. With the second strategy we have obtained induced hepatocytes (iHeps) directly from human fibroblasts (homozygous for p.I244T) by overexpression of hepatocyte specific transcription factors and a hepatocyte defined culture media. From either strategy, we have obtained HLCs and iHeps expressing hepatocyte markers, including albumin expression and glycogen storage, that are being used to study the biology of PH1 *in vitro.* Moreover, we are also testing genome editing strategies for the genetic correction of the cells using CRISPR/Cas9 systems, that after differentiation to HLCs or iHeps would provide new tools for improved cell therapy approaches.

246. Ex Vivo Liver-Directed Gene Therapy Using CRISPR-Cas9-Mediated Genome Editing in Mice

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Hereditary tyrosinemia type 1 (HT1) is an autosomal recessive disorder caused by deficiency of fumarylacetoacetate hydrolase (FAH). A number of animal models of HT1 have been previously described, including our group's generation of the first large animal model of HT1. Using this preclinical porcine model, ex vivo lentiviral-mediated *FAH* gene addition in autologous hepatocytes resulted in complete correction of the metabolic disorder. However, a long term goal of our lab will be the investigation into the efficacy and safety of precise ex vivo genome editing to treat inborn errors of metabolism. To that end, in this study we plan to test whether ex vivo CRISPR-Cas9-mediated gene editing in primary hepatocytes can be achieved in a mouse model of HT1.

First, to achieve high transduction efficiencies of the target hepatocytes, we chose to utilize lentiviral (LV) and adeno-associated viral (AAV) vectors to deliver both the Cas9 nuclease and target guide RNA, as well as the homology repair template, to mouse hepatocytes ex vivo. Transduction efficiencies of hepatocytes with control AAV and LV vectors expressing GFP was determined by microscopy and flow cytometry. Second, guide RNAs were designed to target the locus surrounding the disease-causing SNP in exon 8 of the mouse *Fah* gene for use in conjunction with *Staphylococcus aureus* (AAV-delivery) or *Streptococcus pyogenes* (LV-delivery) of Cas9. Oligonucleotides were cloned and ligated into AAV and LV vectors. AAV.Cas9.sgFAH and LV.Cas9.sgFAH vectors were used to transduce HT1 cells and each guide was investigated for its proficiency to induce double strand breaks at the target locus by harvesting cell pellets, extracting DNA, and analyzing by PCR and restriction enzyme analyses. Third, for repair of the induced double strand break, we designed homology repair templates spanning the region surrounding the target disease SNP that, in addition to restoring *Fah* splicing by correcting the mutation, would also disrupt the PAM sequence and thus inhibit repeated Cas9 cutting at the locus. In vivo AAV delivery to HT1 mice with repair templates was performed to determine if each homology repair template alone was capable of homology directed repair (HDR) without its CRISPR-Cas9 counterpart. Fourth, ex vivo CRISPR-Cas9-mediated gene editing of a mouse model of HT1 is dependent not only on effective transduction of target hepatocytes with both the CRISPR-Cas9 system and homology repair template but also efficient HDR in those traduced cells. Consequently, manipulation of donor and/or recipient hepatocytes may be necessary for efficient HDR due to the nondividing status of most hepatocytes in adult mice. Ongoing work is exploring the feasibility of stimulating homologous repair in each of these target populations. Finally, the functionality of CRISPR-Cas9 mediated-ex vivo gene therapy will be tested in HT1 mice. Hepatocytes will be harvested from donor HT1 mice via retrograde collagenase perfusion of the liver followed by transduction of primary hepatocytes with AAV or LV vectors delivering Cas9 nuclease, target guide RNA, and the homology repair template. Syngeneic HT1 mice will then receive intrasplenic transplantations of transduced primary hepatocytes and will be monitored thereafter for generation of functional FAH hepatocytes through phenotype characterization, biochemical characterization, and immunohistochemical analyses.

Overall, successful ex vivo CRISPR-Cas9-mediated gene editing of primary hepatocytes in a mouse model of HT1 would create the opportunity to examine the application of precise genome editing to treat other inborn errors of metabolism as a future alternative to liver transplantation.

247. Pharmacological Inhibition of Fumarylacetoacetate Hydrolase in Human Hepatocytes

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In vivo selection of gene-targeted hepatocytes is an attractive approach for enhancing the therapeutic benefit of liver-directed gene therapies. We previously reported a universal selection system in which a therapeutic transgene was coexpressed with a short hairpin RNA (shRNA) that conferred resistance to drug-induced toxicity. In this system, toxicity was achieved with 4-[(2-carboxyethyl)-hydroxyphosphinyl]-3-oxobutyrate (CEHPOBA), a small-molecule inhibitor of fumarylacetoacetate hydrolase (FAH). Previous work in our lab has shown that CEHPOBA administration in mice results in a hepatic gene expression profile similar to that observed in severe genetic FAH deficiency. Here we test the effects of CEHPOBA in human hepatocytes. Chimeric mice highly repopulated with human hepatocytes were treated daily with CEHPOBA for two weeks. Control chimeric mice were co-administered NTBC to protect from damage related to FAH inhibition. Liver mRNA was extracted for global expression analysis, and normalized human RNA sequence read counts were compared between the two treatment groups. Relative to the NTBC/CEHPOBA-treated group, human transcripts from CEHPOBA treatment alone showed similar patterns to those observed previously in WT mice. Notably, there was significant up-regulation in genes for oxidative damage (Nqo1) and DNA damage (Rad51). Markers of hepatocyte proliferation were elevated (Afp, Yap1) and a cell proliferation inhibitor, Txnip, was down-regulated. Of particular interest, p21 was up-regulated. In Fah -/- mice, previous work showed that p21 up-regulation is essential for the growth advantage of transplanted cells. These results show the hepatotoxic effects of CEHPOBA in human hepatocytes appear similar to those observed in mouse hepatocytes. These data suggest CEHPOBA could be an effective selective agent for use in human gene therapies.

248. Editing out Five *SERPINA1* Paralogs to Create a New Mouse Model of Genetic Emphysema

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Alpha-one antitrypsin (AAT) deficiency is a common autosomal co-dominant genetic disorder. This condition affects 1:2500 individuals of European ancestry, leading to the development of lung and liver disease. Within North American and Northern European populations, an estimated 4% of individuals are carriers of mutant alleles. AAT deficiency presents with an emphysema phenotype in the lungs of older subjects. AAT deficient subjects can also suffer from liver disease of varying severity; however, lung disease is the principal cause of death. AAT is a protease inhibitor predominantly synthesized in the liver that belongs to the serine protease inhibitor (serpin) family. Upon secretion into the blood stream, AAT enters the lungs where it inactivates excess neutrophil elastase, thereby preventing damage to the alveoli. Mutations of the *SERPINA1* gene can lead to reduced serum levels of AAT and decreased protein functionality, allowing for unrestricted elastin breakdown, pulmonary inflammation and eventual emphysema. Currently, an animal model simulating the lung condition does not exist, which severely limits the development of therapeutics. This is due to the higher genomic complexity of mice compared to humans. Indeed, due to amplification events, C57BL/6 mice have five *Serpina1(a-e)* genes that are homologous to human *SERPINA1*. To address this, we generated a quintuple gene knockout using the CRISPR/Cas9 system via zygote microinjection. Three founding lines were established, in which all five copies of the gene are disrupted. Previously, we showed that mice from all three lines demonstrated absence of hepatic and circulatory AAT protein as well as a reduced capability to inactivate neutrophil elastase. We had also established that the knockouts develop emphysema in response to a two-hit, two-week lipopolysaccharide challenge, following which the mice recapitulated pulmonary mechanics characteristics of the human lung disease including increased compliance. Lung morphometry was affected as well, further supporting the results. Since then, the genomic sequence of the three founding lines has been established by large-insert targeted PacBio genome sequencing. Moreover, the transcriptome of two of the lines has been sequenced and analyzed for off-targets. In addition, knockout mice were aged and their pulmonary mechanics tested to determine whether they spontaneously develop emphysema. The data demonstrates that it is the case, with first signs detectable at 35 weeks of age, and clearly established emphysema at 50 weeks of age. Finally, preliminary results of a six-week smoke exposure challenge, a well-known disease accelerator in patients, will be presented. Further, the ongoing generation of a single copy, full-length Z variant of human *SERPINA1* and future crossing with the knockout line will bring to the field the ultimate α-1 antitrypsin model.

249. Clinical and Translational Studies of Alpha Mannosidosis, a Prototypical Lysosomal Storage Disease

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Alpha-mannosidosis is a prototypical lysosomal storage disease characterized by deficiency of the enzyme lysosomal acid alpha-mannosidase and caused by mutations of the LAMAN gene. This condition is inherited as an autosomal recessive trait with an estimated minimal prevalence of 1: 492,000. Low levels or absence of alpha-mannosidase leads to abnormal accumulation of N-linked oligosaccharides in the brain cells of affected individuals. Delivery

of alpha-mannosidase to the brain is required for treatment of this condition, since it is primarily a neurological disorder. Reversal of central neural storage and ataxia after high dose recombinant enzyme intravenous replacement therapy has been reported in alpha-mannosidosis mice. However, inefficient transport across the blood-brain barrier and the need for repeated infusions due to the limited half-life represent significant drawbacks to recombinant enzyme replacement therapy for human clinical application. Apart from very early bone marrow transplantation, there are no other current or prospective treatments that offer hope for correction of the neurocognitive effects. We are pursuing a collaborative clinical and translational project to better characterize alpha-mannosidosis and develop improved diagnostic and therapeutic regimens, including choroid plexus-targeted viral gene therapy. The choroid plexuses are highly vascularized, slowly dividing structures continuous with the ependymal cells lining the brain ventricles that project into the ventricular cavities, and secrete cerebrospinal fluid (CSF), and various proteins. In a dose-ranging study using recombinant AAV serotypes of variable tropism (AAV4, 5, 6 and 9) we compared the biochemical/pathological effects, and safety profiles in a mouse model of alpha-mannosidosis. On administration to the CSF, serotypes 4, 5 and 6 selectively transduced the choroid plexus epithelia, AAV6 most robustly. In addition to choroid plexus, recombinant rAAV9-treated animals showed pan-neuronal transduction. All serotypes resulted in higher LAMAN enzyme activity throughout the brain, globally, often to normal levels in cerebral cortex, implying secretion of the transgene product into CSF and uptake by neuronal cells.

In a concurrent natural history study, we noted distinctive abnormalities on brain magnetic resonance spectroscopy, and magnetic resonance imaging of lumbar spine in four adult patients with alpha-mannosidosis. Distinctively elevated levels of αMan(1-3)-αMan(1-6)-βMan(1-4)-N-acetyl glucosamine were noted in blood, urine, fibroblasts, and dried blood spots from affected individuals, potentially relevant to future newborn screening. Biochemical and proteomics evaluation of CSF from patients is in progress, to establish a CSF biomarker for potential use in a future first-in-human viral gene therapy trial for this illness. The optimal AAV serotype for human application remains debatable. Based on the mouse results, we will begin to address this question by evaluating AAV6 in a large animal (cat) model of alpha-mannosidosis.

250. Diversion Towards Non-Toxic Metabolites by Gene Transfer for Therapy of Primary Hyperoxaluria Type 1

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Primary hyperoxaluria type 1 (PH1) is an inborn error of liver metabolism due to mutations of the *AGXT* gene encoding the peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT) which catalyzes the conversion of glyoxylate to glycine. In PH1 patients, glyoxylate cannot be converted into glycine and is instead oxidized to oxalate, resulting in systemic oxalosis with deposition of insoluble calcium oxalate in kidneys and in other tissues. Early-onset kidney failure and systemic tissue damage are the consequences of systemic oxalosis. Combined liver/kidney transplantation is the only therapy available to prevent disease progression and mortality. We investigated whether helper-dependent adenoviral (HDAd) vector-mediated hepatic overexpression of glyoxylate reductase/hydroxypyruvate reductase (GRHPR), that converts glyoxylate into glycolate, will steer glyoxylate towards alternative pathways to diminish oxalate production in $Agxt^{-/-}$ mice. Intravenous injection of HDAd-GRHPR indeed resulted in significant reduction of hyperoxaluria and concomitant increase of serum glycolate that was not associated with signs of toxicity. Glutamate-pyruvate transaminase (GPT) in the cytosol catalyzes the transamination of glyoxylate using glutamate and alanine as amino-group donors. The intravenous injection of HDAd vector expressing murine GPT (HDAd-GPT) steered glyoxylate towards transamination and resulted in sustained reduction of hyperoxaluria in $Agxt^{-/-}$ mice. Interestingly, co-administration of HDAd-GRHPR and HDAd-GPT resulted in further reduction and normalization of hyperoxaluria. In summary, we show that metabolic diversion towards non-toxic metabolites has potential for treatment of PH1 and potentially other forms of hyperoxalurias, both primary and secondary. In addition, this study shows that HDAd vectors can be used to functionally validate therapeutic enzyme targets in inherited metabolic diseases.

251. Tol2-Mediated Transgenesis as a Tool for Tissue-Specific Rescue and Assaying Potency of Human Gene Therapy Vectors in the Zebrafish Model of Methylmalonic Acidemia

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Transgenesis is an effective way to rescue animal models of metabolic disorders. However, in mammals its efficiency is limited by low genome integration, germline transmission of novel alleles, and poor survival rates. We establish the utility of Tol2-mediated transgenesis to test a human *MUT* cDNA to rescue zebrafish with a severe form of methylmalonic acidemia (MMA). We used the Tol2Kit vector set to efficiently generate constructs and to enable the modular exchange of promoters to produce tissue-specific cassettes that integrate with a cis-GFP marker expressed in the heart. Using zinc finger mutagenesis, we created a zebrafish model of MMA. This model features a 10 basepair deletion designated mut^0 due to the substitution of a tyrosine with a premature stop codon. By 22 days post fertilization (dpf), mut^0 fish display a 62% reduction in length relative to their wild type siblings ($p<0.001$) and show 100% mortality ($p<0.05$). Tissue analysis reveals high levels of methylmalonate and 2-methylcitrate ($p<0.05$), two biochemical hallmarks of MMA. Histological analysis of 15 dpf mut^0 fish demonstrates clearance of mitochondrial matrix in the gills, similar to the renal proximal tubular epithelial abnormalities seen in patients. Further, eosinophilic inclusions in hepatocyte cytoplasm suggest the presence of megamitochondria seen in MMA patients. The metabolic perturbations, mortality, and mitochondrial abnormalities in the mut^0 fish recapitulate severe symptomatic findings seen in mice and humans, suggesting that transgenesis could mitigate mut^0 fish phenotypes to allow for further study and simultaneously assay the potency of

human gene therapy constructs. To establish the transgenic method, we constructed a cassette with ubiquitous *MUT* expression designed to rescue the *mut*^{0} phenotype. Tol2Kit vectors were used to express the human *MUT* gene (*hMUT*) under control of the zebrafish beta-actin (*actb1*) promoter. The construct also contained the *cardiac myosin light chain 2 (cmlc2):egfp* sequence antiparallel to the *actb1:hMUT* transcript to screen for integrants by GFP. We injected 73 embryos from a *mut*$^{+/-}$ x *mut*$^{+/-}$ mating. At 48 hours post-fertilization, 20 embryos with GFP expression in the myocardium were isolated from clutchmates. hMUT expression analysis is currently in progress. Tol2-mediated transgenesis is a promising technique to achieve highly efficient integration. In our study, we observed 27% GFP^{+} animals compared to <10% typically seen in traditional mouse transgenesis (DeMayo et al., 2012). Screening of embryos by GFP allowed for rapid selection of integration events without genotyping. Taking advantage of the flexibility of the Tol2kit system, we plan to generate vectors that drive tissue-specific expression of human *MUT* and further characterize the effect of *mut* deficiency in organs affected by MMA. To this end, we are pursuing delivery of *hMUT* under control of the *503unc* promoter to create a hypomorphic MMA model with expression specific to skeletal muscle in order to study liver mitochondrial pathology and test AAV constructs for therapeutic potency by cassette insertion into Tol2Kit vectors.

252. Metabolic Pathway Reprogramming: A Novel Therapeutic Approach for Liver Disease

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We recently developed a new therapeutic concept called *metabolic pathway reprogramming*, which couples the power of CRISPR/Cas9 technology with a strategy from pharmacology, namely, to inhibit a metabolic pathway rather than directly edit a disease-causing gene. We demonstrate the efficacy of this approach by using CRISPR/Cas9 to convert lethal type I tyrosinemia into benign type III tyrosinemia in mice. In contrast to small molecule drugs, genome editing is sequence specific and neither its therapeutic potential nor its side effects can be evaluated properly in conventional animal models. To this end, we developed novel xenograft models for metabolic liver disease, which should allow us to properly validate this therapy in the context of primary human cells. We will present the xenograft platform and ongoing research applying metabolic pathway reprogramming *in vivo*.

Oligonucleotide Therapeutics I

253. A Dominant-Negative COL6A1 Pseudoexon Insertion Is Skippable Using Splice-Modulating Oligonucleotides

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Collagen VI-related dystrophies (COL6-RD) are a group of often severe, congenital-onset neuromuscular disorders with no effective treatment. Our group has recently identified a new, and unexpectedly common, *de novo* deep-intronic mutation in the collagen VI alpha 1 (*COL6A1*) gene, associated with severe clinical manifestations. This deep-intronic mutation activates the insertion of an in-frame pseudoexon at the N-terminal end of the triple-helical domain, a location where mutations typically exert dominant-negative effects. Here we further investigate the mechanism of action of this mutation, and we assess a potential exon-skipping intervention. In samples from patients carrying the *COL6A1* pseudoexon mutation, we found that the expression levels of the pseudoexon-containing transcripts were 26.0% in muscle tissue, and 9.0% in skin-derived fibroblasts; significantly lower than what would have been expected from a heterozygous mutation (50%). Using minigene assays, we demonstrated that this intronic mutation creates the 5' donor splice site that activates the pseudoexon insertion, but we found that wild-type splicing still occurred in a certain proportion of transcripts generated from the mutant allele, likely explaining the low pseudoexon expression levels. We have validated this finding in patients' skin-derived fibroblasts, using a synonymous polymorphism located in *cis* of the mutation to track the allelic origin of splicing isoforms. Given the severity of the clinical phenotype associated with this mutation, we investigated whether the pseudoexon-containing alpha chains exert a strong dominant-negative effect on collagen VI assembly. Evidence from non-denaturing (native) immunoblotting, cultured cells' matrix immunofluorescence, and rotary shadowing electron microscopy suggest a dominant-negative mechanism of action for the *COL6A1* pseudoexon. Pseudoexon mutations are potential targets for exon-skipping using splice-modulating antisense oligonucleotides (ASOs). We screened a series of phosphorothioate morpholinos (PMO) and 2'O-Methyl (2'O-Me) ASOs on the minigene assays. We found that pseudoexon-internal ASOs had higher activity than ASOs targeting the splice acceptor or the mutant splice donor. In patients' skin-derived fibroblasts, treatment with pseudoexon-internal PMOs suppressed the incorporation of the pseudoexon in the transcript, reducing its levels by 85% at the highest dose, and blunted the dominant-negative

effect, as evidenced by the improved morphology of collagen VI microfibrils in the matrix. Given that the *COL6A1* intronic mutation appears to be common and has severe clinical consequences, our proof-of-principle demonstration of the successful application of splice-modulating oligonucleotides to suppress the pseudoexon has important implications for the development of therapies for COL6-RD.

254. Imaging LICA Oligo Rescue of RNA Toxicity in Live Mice with Myotonic Dystrophy

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Objective: To compare the efficacy and potency of LIgand-Conjugated Antisense (LICA) oligos vs. unconjugated ASOs to reduce or eliminate RNA toxicity in a transgenic mouse model of myotonic dystrophy type 1 (dystrophia myotonica; DM1).

Background: DM1 is an autosomal dominant muscular dystrophy caused by expression of an expanded CUG (CUG^{exp}) mRNA that has toxic gain-of-function activity, including mis-regulated alternative splicing of pre-mRNA. The HSALR transgenic mouse model of DM1 expresses a human ACTA1 transgene that contains a CUG^{exp} mRNA and features mis-splicing similar to DM1 patients. Mis-splicing of ATP2A1 involves the largest change from normal to DM1 that we have identified. In a previous study, subcutaneous injection of a unconjugated 2′ methoxyethyl (MOE) gapmer ASO targeting ACTA1 transcripts in HSALR mice induced knockdown of CUG^{exp} RNA through the RNase H pathway, resulting in body-wide correction of myotonia and mis-splicing (Wheeler, et al., 2012). LICA chemistry adds specific conjugates to ASOs that are designed to increase drug uptake. In a recent clinical trial, a LICA oligo targeting APO(a) in the liver was several-fold more potent than the unconjugated parent ASO, enabling a >10-fold lower dose and improving tolerability (Viney, et al., 2016).

Methods: We used TR;HSALR "therapy reporter" bi-transgenic mice. The TR transgene in this model consists of DsRED and GFP in mutually exclusive reading frames. Expression of DsRED or GFP is determined by splicing of an upstream ATP2A1 minigene and restricted to skeletal muscle. In this system, inclusion of ATP2A1 exon 22 results in DsRED expression, while exclusion of exon 22, as in DM1, results in GFP expression. Quantitation of the DsRED/GFP ratio by in vivo fluorescence imaging and spectroscopy enables determination of splicing outcomes independent of expression level. ASOs were an unconjugated MOE gapmer targeting ACTA1 and a LICA modified version of the same ASO. We delivered drugs by subcutaneous injection (25 mg/kg twice weekly for 4 weeks) and monitored splicing outcomes in live mice by weekly in vivo fluorescence imaging and spectroscopy.

Results: Beginning at Day 7, DsRED/GFP ratios in LICA oligo-treated mice were greater than in mice treated with unconjugated ASO. Peak ratios in mice treated with LICA oligos were evident by Day 28, but took 42 days in unconjugated ASO-treated mice. RT-PCR analysis confirmed correction of splicing in muscle tissue of ASO-treated mice. Histology showed no evidence of toxicity. Subcutaneous injection of saline had no effect.

Conclusions: LICA gapmer oligos achieve efficient target knockdown in skeletal muscle, while the more rapid onset of action suggests greater target engagement than unconjugated ASOs. Non-invasive fluorescence measurements enable convenient estimation of ASO pharmacodynamic properties in muscle while limiting the number of mice needed. These data support further development of LICA technology for treatment of DM1.

255. Significant Enhancement of Hepatitis B Virus (HBV) Suppression with Standard of Care Drugs Following Co-Administration of a DNA-Directed RNA Interference Agent in a Chimeric Mouse Model

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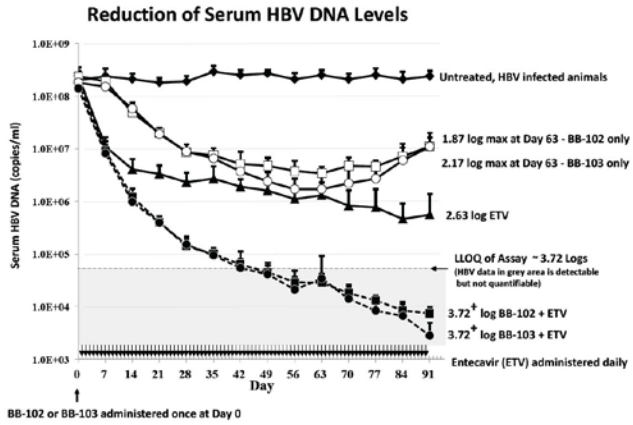
Introduction: BB-101, BB-102 and BB-103 are recombinant AAV8 vectors designed to treat HBV infection using RNA interference following a single administration. Each construct expresses steady state levels of three anti-HBV shRNAs to simultaneously target three well-conserved sequences on HBV viral RNA. BB-101 is comprised of a single stranded AAV that uses modified Pol III promoters for reduced shRNA expression. BB-102 is a self-complementary variant of BB-101. BB-103 embeds the siRNA into miRNA backbones allowing for efficient and natural entry into RNAi pathways thereby conferring the ability to use wildtype Pol III promoters for high levels of expression. The goal of this study was to assess if these vectors, when used in combination with current standard of care drugs, could have a significant impact on HBV viral parameters. **Methods:** PXB mice have chimeric livers comprised of at least 70% human hepatocytes permitting sustained HBV infection. The ddRNAi agents were administered once, at the beginning of treatment, and anti-HBV activity was monitored over the course of 13 weeks by following serum HBV DNA, HBsAg and HBeAg weekly. In combination studies, a single dose of ddRNAi vectors was administered daily with a nucleoside reverse transcriptase inhibitor entecavir (ETV) or pegylated interferon (PegIFN), administered twice a week for the duration of the study. **Results:** As a monotherapy, BB-103, BB-102 and BB-101 treatment yielded a maximum drop of serum HBV DNA levels by 2.17 log, 1.87 log and 0.95 log respectively. Modest rebounds of HBV DNA levels were noted following 56 days of treatment. Treatment with only daily ETV resulted in a 2.63 log drop in serum HBV DNA levels. Remarkably, a single dose of BB-103 and BB-102 in combination with daily ETV dropped the serum HBV DNA levels well below 3.72 log, the lower limit of quantification (LLOQ) of the assay. Though not accurately quantifiable, the viral burden continued to diminish until the end of the 91 day experiment (Figure 1). BB-103 + ETV and BB-102 + ETV dropped HBsAg levels, a known contributor to immunosuppression and HBV chronicity by 2.14 log and 1.86 log. ETV as a monotherapy dropped HBsAg levels by 0.46 log. BB-103 + ETV and BB-102 + ETV dropped HBeAg levels by 1.90 log and 1.42 log respectively. ETV as a monotherapy dropped HBeAg levels by 0.37 log. Co-treatment of mice with PegIFN and the ddRNAi compounds led to less substantial drops, perhaps reflecting on the putative mechanism of action of interferon. **Conclusions:** Using a chimeric mouse model, we demonstrate that the anti-viral efficacy of

standard of care drugs to treat HBV can be significantly enhanced by a single dose of a vector that produces steady state levels of anti-HBV shRNAs. We conclude that the addition of ddRNAi compounds to SOC drug combinations may offer a promising path forward for the treatment of HBV.



256. Baculovirus-Mediated Mir-214 Suppression Shifts Osteoporotic ASCs Differentiation Towards Osteogenesis and Improves Osteoporotic Defects Repair

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Osteoporotic patients often suffer from bone fracture but its healing is compromised due to impaired osteogenesis potential of bone marrow-derived mesenchymal stem cells (BMSCs). Here we aimed to exploit adipose-derived stem cells from ovariectomized (OVX) rats (OVX-ASCs) for bone healing. We unraveled that OVX-ASCs highly expressed miR-214 and identified 2 miR-214 targets: CTNNB1 (β-catenin) and TAB2. We demonstrated that miR-214 targeting of these two genes blocked the Wnt pathway, led to preferable adipogenesis and attenuated osteogenesis, undermined the osteogenesis of co-cultured OVX-BMSCs, enhanced exosomal miR-214 release and altered cytokine secretion. As a result, OVX-ASCs implantation into OVX rats failed to heal critical-size metaphyseal bone defects. However, using hybrid baculoviruses expressing miR-214 sponges to transduce OVX-ASCs, we successfully suppressed miR-214 levels, activated the Wnt pathway, upregulated osteogenic factors β-catenin/Runx2, downregulated adipogenic factors PPAR-γ and C/EBP-α, shifted the differentiation propensity towards osteogenic lineage, enhanced the osteogenesis of co-cultured OVX-BMSCs, elevated BMP7/osteoprotegerin secretion and hindered exosomal miR-214/osteopontin release. Consequently, implanting the miR-214 sponge-expressing OVX-ASCs tremendously improved bone healing in OVX rats. Co-expression of miR-214 sponge and BMP2 further synergized the OVX-ASCs-mediated bone regeneration in OVX rats. This study implicates the potential of suppressing miR-214 in osteoporotic ASCs for regenerative medicine.

257. Optimization of Antisense Oligonucleotide for Skipping Human Dystrophin exon 45, 51 and 53 in Duchenne Muscular Dystrophy Patient-Derived Fibroblasts

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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. We have chosen to target 3 dystrophin exons, ex45, ex51, ex53 for AO drug development to search for the most effective morpholino (PMO) for the correction of the relevant DMD mutations. 30 PMO sequences targeting the three exons have been screened in GFP report cells, NHM, and hDMD mice by i.m. i.v.injection to identify PMO drugs of maximal skipping potency. Each 2 PMOs sequences targeting exon 45, exon 51 or exon 53 were able to skip the targeted exons with 30% or higher efficiency in most of mice muscles by systemic delivery i.v. injection. In this study, we validated optimized PMO sequences in patient-derived fibroblasts. Firstly, we established fibroblast cell cultures from skin biopsies taken from different deletion-specific subgroups of DMD patients predicted to be suitable for therapeutic targeting Ex45, Ex51, and Ex53. We then tested skipping potency of optimized PMO sequence drugs for each exon in the patient-derived fibroblasts cell cultures. We found that optimized PMO sequences for each exon showed relatively similar skipping potency in fibroblast cell cultures from each of the DMD mutation classes, although considerable variation is observed within the same subgroup of patient population. In future, we will test the murine sequence counterparts of the optimized AO drugs (1 for each exon) to skip ex51 and ex53 for histological, functional, and biomarker rescue in the mdxE52 mouse model.

258. Nucleolin Negatively Regulates Cellular Uptake of Phosphorothioate-Modified Splice-Switching Oligonucleotides and Splicing Efficiency in a Variety of Cells

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Duchenne muscular dystrophy (DMD), the most common form of muscular dystrophy, is caused by lack of dystrophin. One of the most promising therapeutic approaches for DMD is splice-switching oligonucleotides (SSOs)-mediated elimination of frame-disrupting

mutations by exon skipping. Phosphorothioate (PS) modification further increases the stability of SSOs and enhances their binding affinity to complementary RNAs. However, the interaction of PS-SSOs with other cellular proteins and the mechanism of splicing regulation are not well understood. Here, we confirmed that high splice-correction activity depends on PS inclusion in 2′-O-Methyl in C2C12 myotubes using different reporter cell lines. Using "photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation" and a subsequent proteomic approach, we identified several SSO-binding proteins including Nucleolin (Ncl), a nucleolar protein that is involved in protein shuttling between the nucleus, cytoplasm, and cell surface through interaction with PS-SSOs. Genetic ablation of Ncl by siRNA in C2C12 myotubes enhanced PS-SSOs uptake but RNA splicing was surprisingly downregulated as evaluated by a luciferase reporter assay. In conclusion, we identified that Ncl physically binds to PS-SSOs, which facilitates the cellular uptake and nuclear distribution of PS-SSOs but downregulates RNA splicing, thus highlighting that Nucleolin-based uptake may be a good target to enhance the efficacy of PS-SSO-based RNA modulation in neuromuscular, hepatic and bone diseases.

259. Spontaneously Loaded Extracellular Vesicles as Carriers for Delivery of Immunotherapeutic Nucleotides

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Oligonucleotide therapeutics (ONTs) can overcome limitations of small molecule inhibitors in targeting of many undruggable transcription factors, such as Signal Transducer and Activator of Transcription 3 (STAT3). However, targeted delivery and short circulatory half-live are still major hurdles in their clinical application. We previously described an original strategy for cell selective delivery of STAT3 inhibitors, in the form of siRNA or decoy DNA, to certain immune and cancer cells using conjugates with TLR9 ligands, CpG oligonucleotides. The CpG-STAT3 Inhibitors (CSIs) showed efficacy in systemic administration against hematological malignancies. To enable use of CSIs for delivery into the microenvironment of solid tumors, such as glioblastoma (GBM), we developed a method of spontaneous encapsulation of the new generation CSI, CpG-STAT3 antisense oligonucleotides (ASO), into extracellular vesicles (EVs). We tested several types of cells for EV-producing capacity, including macrophages, various cancer cells and human neural stem cells (NSCs), which are clinically relevant drug delivery vehicles. The unformulated CpG-STAT3ASO was quickly internalized by various TLR9+ cells but due to excellent stability (T1/2 = 102h in human serum), it resisted lysosomal degradation. Instead, targeted cells secreted EV-encapsulated CpG-STAT3ASO for up to 3 days after internalization. After establishing optimal loading conditions, the EV(CpG-STAT3ASO) were isolated by ultracentrifugation from cultured media, then fully characterized as for vesicle size using Nanosight measurements, expression of membrane antigens and loading efficiency using fluorescent assays and cytofluorymetry. We found that ~80-95% of isolated vesicles were successfully loaded with CpG-STAT3ASO and were characterized by surface staining for CD9-CD63+CD81+ and an average diameter of 125nm. Isolated fraction of EV(CpG-STAT3ASO) were able to transfer biologically active content into glioma target cells (U87, U251) as well as into immune cells. The encapsulation of CSIs did not prevent target gene silencing or TLR-dependent NF-κB activation. In our preliminary biodistribution studies, EV-encapsulation improved delivery of fluorescently-labeled CpG-conjugates to target cells in various organs after intravenous injections into mice compared to equal amount of naked oligonucleotide. We demonstrated the feasibility of using spontaneously EV-encapsulated CSIs for improving delivery of ONTs to TLR9+ immune and cancer cells, with simultaneous preservation of biological activity of encapsulated drug. Our further studies will verify whether EV-encapsulated ONTs will allow for improved penetration of solid tumors and their distant metastases.

260. The Essential Role of Satellite Cells and Macrophages in Efficient Delivery of Antisense Morpholinos into Dystrophic Muscle Fibers

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Duchenne muscular dystrophy (DMD) is a progressive myopathy, characterized by persistent muscle degeneration and regeneration, inflammation, and fibrosis. Exon skipping is a promising therapeutic strategy for DMD, employing antisense oligonucleotides (AO) to exclude disruptive exons from the mature mRNA transcript, so as to elicit production of a internally-truncated, partially-functional dystrophin protein. However, clinical trials of systemically delivered AO have produced variable exon skipping and patchy dystrophin expression mirroring the sporadic nature of dystrophic pathology. To identify the factors leading to this variability, we investigated the influence of myofiber regeneration on drug uptake and exon skipping by treating dystrophin-null *mdx* mice with antisense morpholino (PMO) together with staggered pulses of bromodeoxyuridine. This enabled us to define the precise stage of myofiber regeneration, relative to systemic PMO delivery, that coincides with optimal drug uptake and exon skipping. Robust PMO localization and elicitation of dystrophin expression was limited to dystrophic lesions regenerating during the 3 days prior to, and concurrent with, PMO administration. Sustained PMO accumulation was exclusive to inflammatory foci where it predominantly entered macrophages, actively differentiating myoblasts and newly forming myotubes. Furthermore, cultured PMO-loaded macrophages showed robust uptake and sustained retention of intracellular PMO, and subsequently released the majority of their PMO content. Together this identifies macrophages as a persisting local source of PMO that is not in direct equilibrium with serum drug levels, thereby extending the duration of local drug availability to the newly forming myofibers via differentiating and fusing myogenic precursors. We conclude that the observed variability in PMO-induced dystrophin expression reflects the limitations on drug delivery imposed by the need for three associated events: first, release of PMO from the

vasculature into the muscle interstitium by inflammatory exudation associated with myopathic lesions; second, sustained PMO storage within the macrophage reservoir; third, fusion of PMO-loaded myoblasts into the newly repairing muscle fibers. Identification of these pathophysiological factors that regulate the efficiency of PMO delivery accounts for the observed clinical variability and suggests strategies to improve this therapeutic approach to DMD.

261. Oligonucleotides Targeting CTG Repeats in DNA Down-Regulate Huntingtin

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We have developed a new therapeutic approach by targeting the trinucleotide DNA repeat in Huntington's disease (HD). This is in contrast to most other oligonucleotide (ON) therapeutics which are antisense for RNA. HD is a fatal, neurodegenerative disorder in which patients suffer from mobility, psychological and cognitive impairments. Existing therapeutics are only symptomatic and do not significantly alter the disease progression or increase life expectancy. HD is caused by expansion of the CAG trinucleotide repeat region in exon 1 of the Huntingtin gene (*HTT*), leading to the formation of mutant *HTT* transcripts (mu*HTT*). The toxic gain-of-function of muHTT protein is a major cause of the disease. In addition, it has been suggested that the mu*HTT* transcript contributes to the toxicity. Thus, reduction of both muHTT mRNA and protein levels would ideally be the most useful therapeutic option. Using ONs directly targeting the *HTT* CTG trinucleotide repeat DNA a partial, but significant and possibly long-term, HTT knock-down of both mRNA and protein was successfully achieved in HD patient derived fibroblasts. Diminished phosphorylation of *HTT* gene-associated RNA-polymerase II was observed, suggestive of altered chromatin and reduced transcription downstream the ON-targeted repeat. Different backbone chemistries were found to have a strong impact on the ON efficiency. We also successfully used different delivery vehicles as well as naked uptake of the ONs, demonstrating versatility and providing insights for *in vivo* applications.

262. Differentiate Mesenchymal Stem Cells

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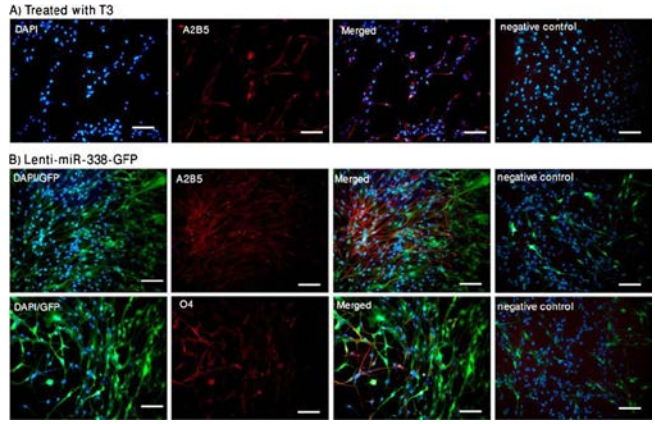
Abstract: Oligodendrocytes (OLs) are responsible for myelin sheath synthesis around nerve fibers in the central nervous system (CNS) that form the myelin sheath of axons. MicroRNAs have a critical role in oligodendrocyte development including cell proliferation, differentiation, and myelin formation. MicroRNA 338 (miR-338) is necessary to promote oligodendrocyte differentiation by repressing negative regulators of oligodendrocyte differentiation. Human endometrial-derived stromal cells (hEnSCs) are a new source of mesenchymal-like stem cells for cell replacement therapy. The hEnSCs, after treating with fibroblast growth factor 2/epidermal growth factor (20 ng/mL) and platelet-derived growth factor (PDGF)-AA (10 ng/mL) for 12 days, were divided in two groups: in the first group, the cells were treated by triiodothyronine (T3), and in the second group, the cells were infected by miR-338-green fluorescent protein-expressing lentiviruses. Six days after infection, the cells were collected and analyzed for the expression of stage-specific markers Nestin, microtubule-associated protein 2, neurofilament-L, oligodendrocyte lineage transcription factor, SRY-box containing gene 10, PDGF receptor alpha, 2',3'-cyclic nucleotide 3' phosphodiesterase, A2B5, O4, and myelin basic protein by immunocytochemistry and quantitative reverse transcription PCR. Result showed that in the infected cells, the expression of pre-oligodendrocyte markers was higher than that of T3-treated cells. The EnSCs can differentiate to oligodendrocyte cells by the overexpression of miR-338, and these cells can be used as a unique source for cell therapy of neurodegenerative disease.



263. Genetic Delivery of Anti-Sense RNAs to Alter mRNA Expression of Tyrosine Kinase Receptors in Human Gliobastoma

Sarah Falotico, Sachin Parikh, Nicole Sivetz, Peter Nekrasov, Imari Patel, Priyal Patel, Kerianne Fuoco, Martin J. Hicks

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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 can lead to uncontrolled growth and proliferation of GBM. Although a great deal is known about the 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 are often utilized to target and silence gene expression, exogenously expressed RNAs are often susceptible to extracellular and intracellular nucleases as well as activation of cellular immunity against foreign nucleic acids. To bypass these degradatory mechanisms, we take advantage of natural noncoding RNA gene architectures and pathways along with an anti-sense targeted approach to alter the expression of tyrosine kinase receptors. We make use of an anti-EGFR polycistronic delivery system to express multiple RNAs directed against EGFR, specifically targeting the transmembrane domain of the EGFR transcript. Gene delivery vectors were transfected into the human GBM cell lines. Results show that our vectors were expressed at high levels with subsequent reduction in EGFR mRNA expression. Future strategies include using the polycistronic delivery mechanism to target multiple tyrosine kinase receptors.

Pharmacology/Toxicology Studies or Assay Development

264. INSPIIRED: A Pipeline for Quantitative Detection of Newly Integrated Genomic Elements

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Integration of DNA into cellular genomes is a unique trait of retroviral replication and transposon multiplication, both of which have been adapted for use in human gene therapy. Identifying the genomic locations of newly integrated elements is important for tracking transduced cells and monitoring potential outgrowth of pathogenic clones. Here we report a quantitative analytical pipeline named INSPIIRED (integration site pipeline for paired-end reads). We optimized ligation-mediated PCR for integration site capture and analysis using the Illumina paired-end sequencing platforms. In this method, genomic DNA is broken initially during library preparation by sonication, followed by ligation of DNA adaptors. These linker/breakpoint sites can then be utilized to infer abundance of individual cell clones, allowing quantification of cells harboring each integration site. We include technology for suppressing recovery of unwanted contaminants, sensitivities or limits of detection, and software for managing alignments, quality control, and integration site data. Using *in-silico* generated integration sites of known positions, we optimized our sample processing parameters by comparison to truth. Because approximately 40% of the human genome is made up of repeated DNA sequence, some integration site sequences align to multiple locations (multihits). We present a novel graph-theory-based method to quantify multihit integration sites and characterize the consequences using *our in-silico* data set and experimental data. We also developed tools for data analysis and visualizations that take advantage of the data obtained. Tools such as interactive heat maps allow for comparison of distributions of integration sites to genomic or epigenetic features, supporting numerous user-defined statistical tests. For summarization, we developed a reproducible report format that considers all collected unique and multihit integration sites for a designated patient and catalogs the sample population structure, longitudinal dynamics, and integration frequency near cancer-associated genes. Examples of the use of this pipeline will be shown from recent gene therapy trials.

265. Improving the Safety of Lentiviral Vector Integration with Chromatin Insulators

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Self-Inactivating (SIN) Lentiviral Vectors (LV) have demonstrated great efficacy and safety in preclinical models and clinical trials. Still, SIN. LVs are not entirely neutral to the cell genome. To reduce the risk of oncogene activation by SIN.LV insertions, Chromatin Insulators (CI) can be included in the LV constructs. To this aim we tested four CI based on the best characterized insulator-protein: the CCCTC-binding factor (CTCF). We cloned each CI in the Long-Terminal Repeats of a SIN.LV with a strong enhancer/promoter and tested the safety profile of these insulated-LVs (CI.LVs) *in vivo* in two sensitized mouse models of full and partial *Cdkn2a* deficiency (n= 156 mice). These models allow measuring vector-induced genotoxicity as accelerated tumor onset vs. Mock and Integration Sites (IS) retrieval from tumors followed by Common Integration Sites (CIS) analysis enable identifying the mechanisms of insertional mutagenesis. In $Cdkn2a^{-/-}$ mice all CI.LVs displayed slight, but not statistically-significant, improvement in the median survival time vs. the uninsulated-SIN. LV. In $Cdkn2a^{+/-}$ mice the median survival time for two CI.LVs was significantly different from the uninsulated-SIN.LV (p-values: 0.0135 and 0.0063) and not different from controls. We analyzed >15000 IS and identified different CIS. In $Cdkn2a^{-/-}$ mice, uninsulated-SIN. LV-induced tumors harbored predominantly activating insertions targeting *Map3k8* oncogene, while mice treated with two CI.LVs had significantly reduced frequency of tumors with *Map3k8* IS. The reduced *Map3k8*-targeting was accompanied in one case by a skewing towards inactivating-integrations targeting tumor-suppressors (*Pten, Rasa1*), as genotoxicity escape mechanism. In $Cdkn2a^{-/+}$ mice we identified different predominant CIS genes by the four CI.LVs, suggesting that this model allows discriminating between more subtle shades of genotoxicity. Interestingly, merging the results from both *in vivo* assays we observed that one CI displayed superior safety profile in terms of significant improvement in the median survival-time and of reduced oncogenic CIS identified. In summary, we validated new human-origin CI able to block SIN.LV genotoxicity *in vivo*. Overall, these data support the use of CI for future gene therapy applications. In line with this, we have recently generated a panel of insulated-SIN.LVs

with the moderate cellular promoter Phosphoglycerate Kinase (PGK), representing a vector design more similar to the one currently exploited in gene therapy clinical trials. The validation of these insulated vectors, by means of transgene expression levels and stability in transduced CD34+ cells transplanted in NSG mice, will help bringing CI closer to the clinics.

266. Development of a Single-Cell PCR Assay to Quantify the Percentage of Cells Transduced with Lentiviral Vectors

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The success of cell-based gene therapy relies on, in part, obtaining a sufficient number of vector-transduced cells in the final drug product (DP). Historical assessments of transduction efficiency have relied on calculating an average vector copy number (VCN) normalized across the population of cells in the DP (expressed as VCN per diploid genome). However, VCN itself does not directly inform on the percentage of cells containing at least one transgene. Moreover, the percentage of transduced, or lentiviral vector (LVV)-positive cells (%LVV+) may be an important attribute of certain gene therapy drug products - particularly for those in which the transgene functions cell autonomously. Measurement of %LVV+ cells can be complicated by the lack of expression of the transgenic protein in the assayed cells, absence of fluorescent reporters in clinical vectors, and/or lack of suitable methods for detection of transgene expression. Here, we report the development and qualification of a single-cell PCR (scPCR) assay to detect individual cells with one or more integrations of LVV sequences; enabling the quantification of the %LVV+ cells in a population (the method does not provide a single-cell VCN value). Using untransduced CD34+ cells, the assay was shown to have a 0.17 % false positive rate. Using a stably-transduced cell line, the assay was shown to have a 5.02 % false negative rate. After transduction, non-integrated LVV DNA was found to decay in ~3 days, with consistent assay readout between 3 and 7 days after transduction. The assay was compatible with a freeze/thaw cycle after transduction. Accuracy was confirmed by transducing cells with a GFP LVV and comparing the percentage of LVV+ cells from scPCR to the percentage of GFP+ cells by FACS ($r^2 = 0.96$) or LVV+ colonies by real time PCR ($r^2 = 0.91$). Assay controls were developed and acceptance criteria determined by retrospective analysis. The established assay acceptance criteria were met 99% of the time across 320 samples tested, regardless of cell type (CD34 or T cell) and LVV transgene. Intra-assay precision was found to have 16 %CV when using a single 96-well plate per sample, and was reduced to 8 %CV when using three 96-well plates per sample, consistent with statistical margin of error of the assay. Linearity was determined across the range of 0-100 %LVV+ cells. The assay performed consistently with as few as 5,000 transduced cells, allowing for enumeration of %LVV+ cells in rare specific cell sub-sets such as phenotypic hematopoietic stem cells (CD34+/CD38lo/CD90+/CD45RA-), while peripheral blood matrix slightly increased the false positive rate but had no effect on assay linearity or precision. Lastly, we compared the results obtained with the %LVV+ assay and the traditional VCN approach. Plotting %LVV+ cells against VCN produces an asymptotic curve that clearly deviates from a Poisson distribution, and %LVV+ cells can be calculated from VCN. Together these data demonstrate the successful development of a scPCR-based assay for %LVV+ cells.

267. Development of a Cell-Based AAV9 Vector Potency Assay for the GAN Phase 1 Clinical Trial

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Recombinant adeno-associated viruses (AAVs) show significant promise to deliver therapeutic genes to treat human diseases and clinical studies using AAV have been initiated for a number of conditions, including Giant axonal neuropathy (GAN). GAN is a rare genetic disease characterized by a progressive motor-sensory neuropathy that results in death typically in the early 20s. A phase 1 clinical trial for GAN is currently underway at the NIH using an AAV vector, scAAV9/JeT-GAN. AAV vectors prepared for clinical research must be thoroughly characterized and meet predetermined standards for safety, purity, potency, and stability. The potency of clinical grade vectors is characterized by both genome concentration and by assays that assess the functional activity of the vector. For the GAN clinical trial, we have developed a potency assay that demonstrates GAN transgene expression following transduction of an appropriate cell line in a dose-dependent manner with the clinical vector. For the assay, AAV9-permissive Lec2 cells are infected with scAAV9/JeT-GAN at a MOI of 10^4 and 10^5 vg/cell for 48 hours and RNA is then isolated from these cells. cDNA is then synthesized, and GAN transgene expression is measured using qPCR analysis. The GAN activity unit (A.U.) is then calculated by dividing the number of GAN transgene copies by the number of Lec2 GAPDH copies and normalized by the infection dose. A previously characterized preclinical grade scAAV9/JeT-GAN (titer = 3.3×10^{13} vg/mL) is assayed in parallel as a reference material for the clinical grade vector. Using this assay, we have tested the potency of the clinical grade scAAV9/JeT-GAN (titer = 1.8×10^{13} vg/mL) vector that was formulated in 1x PBS containing 5% sorbitol at vialing and at 6, 9, 12, and 18 months post-vialing on aliquots that were stored at ≤-60°C. The average potency of clinical grade scAAV9/JeT-GAN was 3.29 (+/- 0.06) A.U. and there were no significant differences between the A.U. of the post-vialing test points. We have also found that when infected at the same dose, the preclinical grade scAAV9/JeT-GAN vector serves as a reliable reference material with an average potency of 3.95 (+/- 0.41) A.U. over the course of testing. A second lot of clinical scAAV9/JeT-GAN vector (titer = 1.7×10^{14} vg/mL) was produced for the GAN trial and vector potency was tested. Using our assay, we found that at vialing the second scAAV9/JeT-GAN vector lot had a potency of 3.32 (+/- 0.18), and no significant difference was found in the A.U. when compared to the first clinical vector lot. Having found that the potency of AAV9 remains stable over a year after vialing and when stored at ≤-60°C, we then asked if the potency is stable when virus is

thawed and stored at 4°C. Using our potency assay, we found that the preclinical grade scAAV9/JeT-GAN vector that was freshly thawed, or thawed and stored at 4°C for either 6, 12, or 18 months, had an average potency of 3.74 (+/- 0.23) A.U. with no decrease in potency over time for vectors stored at 4°C. Overall, we have found that the AAV9 cell-based potency assay to be highly reproducible and reliable for testing the activity of clinical and preclinical vectors.

268. Development and Validation of a GMP Grade Lentiviral Vector for the Gene Therapy of Pyruvate 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. PKD is associated with reticulocytosis, splenomegaly and hepatic iron overload, and may be life-threatening in severely affected patients. To-date, allogeneic bone marrow transplant represents the only curative treatment for severely affected patients but has been employed infrequently. 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. Preclinical gene therapy studies conducted in pyruvate kinase deficient mice have shown the safety and the efficacy of a new PGK-coRPK-Wpre therapeutic lentiviral vector 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 we have developed an optimized GMP-grade lentiviral vector production. The manufacturing is based on the solid phase bioreactor on adherent HEK293T cells with an optimized protocol of calcium-phosphate transfection. The core dowstream process is AEX chromatography followed by centrifugation. Final products were generated with viral titres up to $2x10^9$ Viral Copy Number/ml (VCN/ml) with a high performance measured by p24 ELISA.The system has been scaled up to produce batches from 30-70 L of harvest with no significant loss in viral production yield. These viral batches have been tested for transduction efficiency in healthy cord blood $CD34^+$ cells. Up to 90% colony forming cells tested in semisolid cultures were transduced. Analysis of 14-days liquid cultures showed values between 1 and 3 VCN/cell, demonstrating good transduction efficiency, compatible with a clinical application. Optimization of the transduction conditions -- including cell concentration, multiple rounds of transduction and inclusion of transduction enhancers -- is underway in order to improve reduced the amount of viral vector needed to achieve optimal transduction efficiencies.

269. Comparison of Rapid Sterility Testing Methods (BacT-Alert Microbial Detection System versus MicroBio μ3D System) for Cell Therapy Product Release

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Background: Sterility testing is an essential release criterion for cell therapy products. USP71, a compendial sterility method in the United States, comprising of visual monitoring after a 14-day growth period of product in culture broth, is generally used for release of biologics and cell therapy products in the United States. While USP71 works for most products that are cryopreserved or have a longer shelf life, the turnaround time can be a major limiting factor for release of fresh cell therapy products that have a post manufacturing shelf life of a couple of days if not hours. Often the practice for fresh cell therapy applications is to conditionally release final products for patient treatment prior to final results from USP71. The conditional release allows the product to be used for treatment before its expiry based on negative Gram stain and endotoxin results to provide a level of product sterility assurance. However, to eliminate any concerns to patient safety, rapid sterility methods have the potential to provide equivalent assurance to the USP71 method with results available for fresh cell therapy products before their release for treatment. **Method:** In the current pilot study, a direct comparison of two rapid sterility systems is conducted. These systems are: 1. BacT/Alert Microbial Detection System by BioMériuex, Inc. ("BacT/Alert", a microbial detection system that implements the gas measurement method); and 2. MicroBio u3D System by MicroBio Corporation, Japan (a growth based microcolony imaging system). In-house cell therapy product aliquots were inoculated, each with one of the 6 compendial strains. Duplicate aliquots for each strain were cultured using methods conforming to the respective detection systems, and monitored for post inoculation detection times. Appropriate negative controls were also used in the current study. **Results:** The results of the above study indicated the following:

1. MicroBio u3D System was able to detect microbial growth *(Bacillus subtilis)* as early as 7 hours post inoculation, while detecting growth of all compendial strains within 34 hours. The system however detected false positive in one of the negative control samples.

2. BacT/Alert could detect microbial growth *(Bacillus subtilis)* as early as 16.3 hours post inoculation, and growth of all but one compendial strains were detected within 28 hours. Detection of *Aspergillus niger / brasiliensis* took longer (detected at 56.2 hours) as predicted from previous studies from other groups.

Conclusion: MicroBio u3D System demonstrated higher sensitivity and shorter times in microbial growth detection than that in the in BacT/Alert system. In contrast, BacT/Alert system when compared to MicroBio u3D System had 0 false negative/positive results, and demonstrated higher reliability. MicroBio u3D System could become

a better alternative as a rapid sterility method in terms of speed, but requires further development in methodology for it to become a more steamlined and reliable testing method in the future. In particular, the risk of false positives should be eliminated to avoid unnecessary rejection of fresh products because there is not adequate time to investigate and confirm the false positive before the product expires.

270. Three-Dimensional Co-Culture Bioassay for Screening of Neurotrophic Factor Gene Delivery System for Glaucoma Therapy

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Purpose: The primary purpose was to develop an *in vitro* retinal bioassay that can be utilized as a screening tool to systematically validate and evaluate the functional effects that can be achieved by therapeutic proteins produced from cells transfected with therapeutic protein-encoded plasmid gene delivery systems designed for retinal ganglion cell (RGC) rescue.

Introduction: Glaucoma is the world's second leading cause of blindness that is a result of progressive RGC degeneration. Neurotrophic factor gene therapy is a promising therapeutic approach that aims to address the unmet need in current intraocular pressure reduction focused regimens by providing damaged RGCs with neurotrophic support as a mean of protection and repair. To evaluate the therapeutic potential of brain-derived neurotrophic factor (BDNF) gene therapy for RGC rescue, we have developed a three-dimensional co-culture model to simulate the cellular interaction *in vitro,* comprising of BDNF-expressing astrocyte (trA7) cells and BDNF-responsive oxidatively stressed neuroblastoma (oxSH-SY5Y) cells. Furthermore, molecular quantitation and immunofluorescent imaging methodologies have been incorporated to evaluate the therapeutic potential of gene delivery systems.

Methods: The establishment of the co-culture model involved a multi-step process that assembles trA7 cells cultured in the transwell insert with oxSH-SY5Y cells cultured in the well reservoir. Transfection of A7 astrocyte cells with BDNF-encoding plasmid DNA was carried out using K2® gene delivery system (K2-NPs). Bioavailability and bioactivity of secreted BDNF protein were quantitated using enzyme-linked immunosorbent assay (ELISA), and measured through the combination of immunofluorescent imaging, tracing and profiling of neurites extended from β-III tubulin labelled SH-SY5Y neuroblastoma cells, respectively.

Results: The co-culture model effectively simulates the cellular microenvironment between the transfected "medic" cell population and the "stressed" cell population receiving the therapeutic proteins. By culturing separate populations on either transwell insert or well reservoir, it allows for precise co-culture/separation timing for versatile experimental design. On the other hand, the bioassay also effectively evaluated the therapeutic potential of BDNF gene delivery system. Quantitation of BDNF secreted in the co-culture system demonstrated that trA7 cells produced 3,750.8±251.1, 9,052.6±1391.2 and 10,367.1±390.8 pg/mL of BDNF protein at 24, 48, and 72 hours, respectively. Moreover, through neurite length distribution profiling, ox-SH-SY5Y cells co-cultured with trA7 cells consists of up to 10.8 times more neurites with extended length (>20μm) compared to oxSH-SY5Y cells that were cultured in the absent of trA7 cells. Thus, demonstrating the promising role that BDNF gene therapy holds in RGC rescue.

Conclusion: As the success of a plasmid gene therapy regimen is heavily dependent on factors beyond successful intracellular plasmid entry such as *in situ* produced protein bioavailability and bioactivity, the three-dimensional co-culture bioassay described within could serve as a crucial screening tool in future gene delivery system developments.

271. Development of the Testing Methodologies for Gene Therapeutic Products of Adeno-Associated Virus and Vaccinia Virus Vectors

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Purpose. The purpose of this study was to develop fast and reliable testing methodologies of raw materials, such as cell substrates and virus banks for the manufacture of adeno-associated virus (AAV) vectors and vaccinia virus vectors by a quantitative real-time polymerase chain reaction (qPCR), which then had been further validated to establish the standard operating procedure (SOP) of the developed testing methods.

Methods. For a safety testing of Sf9 cell substrate for the manufacture of AAV vectors, insect cell-specific contamination of Boolarra virus (BoV), Flock house virus (FHV), Nodamura virus (NoV) and Pariacoto virus in Sf9 cell substrates were detected by qPCR. The developed testing method of BoV contamination was further validated based on ICH Q2(R1) to establish the SOP. The fast and reliable quantitation method of vaccinia viral genomes in virus banks for the manufacture of their clinical lot was developed using a qPCR, which had been validated for the standardized method.

Results and Conclusions. AAV vectors have been manufactured using either HEK293 or Sf9 cell substrates. Since production of AAV vectors in Sf9 cells has several advantages of the improved production efficiency, the decreased production cost and the efficient scale-up process. Therefore, we developed the detection method of adventitious insect cell-specific virus contamination, such as Alphanodavirus of FHV, NoV, BoV and PaV. All four virus of FHV, NoV, BoV and PaV have RNA genome, therefore their RNA genome templates of around 90 nucleotides and several pairs of each virus-specific primers were synthesized to develop the quantitative detection methods using a reverse-transcription PCR (RT-PCR). The optimal RT-PCR conditions of the primer concentration, the Taqman probe concentration and the annealing temperature were determined and the developed method to quantitate the contaminated BoV in the cell substrate had been validated based on ICHQ2(R1). The specificity, accuracy, precision, LOD of 942 copies and LOQ of 3,420 copies had been validated to establish the SOP.

Plaque assays have been widely used to measure infectious titers of vaccinia virus vectors, however, a faster and reliable titration method need to be developed. We developed the quantitation method of vaccinia viral genomes using a qPCR. The optimal PCR conditions

were determined using the E9L template plasmid and three gene-specific primer sets. For quantitation of vaccinia viral genomes, the genome DNAs were extracted and included in the qPCR reactions. The developed titration method was then validated based on ICHQ2(R1) of specificity, linearity, range, precision set true value and accuracy to establish the standard method.

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Keywords. adeno-associated virus, vaccinia virus, gene therapy, testing, SOP

272. Profile of Circulating and Neutralizing Antibody Titers Towards AAV8 & AAV9 in Cynomolgus Macaques

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Duchenne muscular dystrophy (DMD) is a monogenic skeletal muscle disease caused by deficiencies in dystrophin, a vital protein for sarcolemma stabilization. Loss of functional dystrophin results in progressive body wide skeletal muscle wasting. Gene therapy approaches are being developed to treat DMD using systemic delivery of recombinant adeno-associated viruses (rAAVs) and encouraging data has been generated treating dystrophic preclinical mouse and dog models. AAV8 and AAV9 are promising candidate AAV serotypes for skeletal muscle diseases, including DMD, due to their muscle tropism. To better predict clinical translation of rAAV gene therapy, non-human primates (NHPs) studies are performed to assess both safety and biodistribution. Pre-existing serum antibodies to AAV capsids, both neutralizing and non-neutralizing, can have consequences for not only patient safety but also impact efficacy by inhibiting viral transduction. Therefore, understanding the frequency of seroprevelance in an animal population is important for predicting resource availability for these important pre-clinical studies. In this analysis, total IgG and neutralizing serum antibodies against AAV8 and AAV9 capsids were measured in 226 juvenile cynomolgus macaques of Cambodian origin. Animals where housed by two different vendors in three different domestic holding facilities. Fourteen animals (6%) of all NHPs screened were considered low for both AAV8 and AAV9 neutralizing (<1:5) and circulating (IgG) (< 50,000 mU/mL AAV9, < 100,000 mU/mL AAV8) antibodies, while sixty-one (27%) of all NHPs screened had neutralizing antibody levels of <1:5 for both AAV8 and AAV9. Seroprevelance among populations were markedly different. In one population, the levels of neutralizing and circulating seronegativity was correlative while the other population more than half of the animals that had neutralizing antibody levels of <1:5 for both AAV8 and AAV9 were moderate to high in circulating antibody levels. Further understanding of pre-existing humoral immunity to AAV capsids in both pre-clinical models and patients will facilitate the advancement of clinical approaches to overcome them.

Targeted Gene and Cell Therapies for Cancer

273. Delivery Cellular Platform Enables Targeted Brain Delivery to Glioblastoma

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BACKGROUND: Cancer renders the endothelium anergic, abrogating T cell homing and hindering the development of effective cell therapy for central nervous system (CNS) malignancies. Successful trafficking of leukocytes relies on a complementary interaction of homing molecules. Lessons learnt from inflammatory brain diseases can give insight into how to overcome the blood brain barrier (BBB) blockade created by cancer. Activated Leukocyte Cell Adhesion Molecule (ALCAM) is a pathological adhesion molecule upregulated in the endothelium of a number of inflammatory/infiltrative CNS diseases, such as multiple sclerosis. Upregulation of ALCAM in these conditions leads to overexpression of Intercellular Adhesion Molecule-1 (ICAM-1) and vascular CAM-1 (VCAM-1); two ubiquitous adhesion molecules that mediate firm leukocyte anchorage thus enabling successful trans-endothelial migration (TEM). Antibodies blocking ALCAM decrease leukocyte access to the brain and are currently being tested in a clinical trial for MS. Our research proposes a cellular platform that regains the access for T cells to brain tumors, through preferential anchorage to ALCAM. **METHODS/RESULTS:** We studied the difference in the dynamic signature of adhesion molecules in the "anergic" brain tumor endothelium and that of infiltrative brain conditions. We found that ALCAM is highly expressed on primary glioblastoma (GBM) endothelium, while the downstream factors ICAM-1 and VCAM-1 are paradoxically downregulated. Moreover, we saw a significant induction of ALCAM but not VCAM and ICAM expression when GBM endothelium was cultured in supernatants from primary GBM cells or in TGFß. Thus, GBM endothelium fails to launch the second wave of adhesion molecules necessary for firm T leukocyte capture and BBB transmigration. We mapped the ALCAM minimal binding region to domain 3 (D3) of CD6 and created an artificial molecule with the intent of creating a novel cellular platform to reverse endothelial anergy, through ALCAM specific binding. The exodomain of this molecule is composed of D3 linked by a stalk, transmembrane domain and an endodomain from CD6, and successfully expressed it on the surface of T cells using retroviral transduction. D3 on the T cell crosslinked to ALCAM on endothelial cells in proximity ligation assays (PLA; <40nm) during TEM. Under shear stress, D3 T cells showed a

global improved ALCAM specific trafficking kinetics: higher capture on ALCAM+ endothelium, rolling with slower velocity, and better TEM. This was not seen on D3 interaction with ALCAM (-) normal endothelial cells or with non-transduced (NT) control T cells. In an ex vivo model of BBB (endothelium/membrane/pericyte), D3 T cells exhibited higher transmigratory ability. We discovered that signaling through the D3 endodomain phosphorylated pZAp70 recruiting Talin that enables LFA-1 (ICAM-ligand) open confirmation, mediating effective TEM. Using TIRF microscopy, D3 T cells had higher F-actin/FAK colocalization at its interface with ALCAM; indicating that D3 mediates better mechanical stabilization of the anchorage and TEM process. Lastly, in an orthotopic model of GBM, D3 T cells homed more and accumulated at the tumor site compared to NT controls. This was confirmed by the specific detection of D3 T cells in brain explants on pathological examination. **CONCLUSION:** We created a cellular platform that enables targeted brain delivery of T cells. It serves as a gateway to the effective cellular therapeutics for brain malignancies but potentially as a delivery system for complex biologics for other pathological conditions.

274. Intrathecal AAV9.trastuzumab for Prophylaxis and Treatment of HER2+ Breast Cancer Brain Metastases

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Breast cancer brain metastases (BCBM) occur in up to 30% of patients with human epidermal growth factor receptor 2 positive (HER2+) primary tumors. Patients survive only 3-24 months after BCBM diagnosis, and available treatments are invasive, toxic, and largely ineffective.[1] Intravenous trastuzumab (anti-HER2 monoclonal antibody (mAb), Herceptin®) extends survival in patients with HER2+ systemic disease, but does not cross the blood-brain barrier (BBB) to treat HER2+ BCBM effectively.[2] Intrathecal (IT) trastuzumab can extend survival in patients with HER2+ BCBM,[3] but this requires frequent IT infusions, which can result in a widely fluctuating pharmacokinetic profile of trastuzumab in CSF that may compromise its therapeutic effect.[4] Gene therapy offers a one-shot solution for mAb delivery across the BBB. Adeno-associated viral vectors, particularly serotype 9 (AAV9), can safely and efficiently deliver exogenous genes to central nervous system tissues after a single IT administration, resulting in constitutive, stable expression of the transgene product.[5] Here, we characterize a xenograft model of HER2+ BCBM using BT474. M1 tumor cells injected stereotactically into the brain parenchyma of Rag1-/- mice. AAV9.trastuzumab was delivered IT as tumor prophylaxis at least 21 days before tumor implantation or as tumor treatment 3 days post tumor implantation. Median survival of mice that received IT AAV9.trastuzumab, either prophylactically or as treatment, was significantly greater than mice that received control treatments (Figure 1). Immunofluorescence microscopy was used to visualize expressed trastuzumab that was bound to tumor cells, and identification of immune infiltrates in tumors was performed by immunohistochemistry. We also employed NK cell and macrophage depletion in our Rag1-/- tumor prophylaxis model, as well as IT AAV9.trastuzumab tumor prophylaxis in NOD *scid* gamma (NSG) mice, to demonstrate that IT AAV9.trastuzumab efficacy depends on the presence of NK cells but not peripheral macrophages. Finally, we evaluated IT AAV9.trastuzumab brain tumor prophylaxis and treatment in an immune-competent 4T1 BALB/c model using 4T1 tumor cells transduced with human HER2. Our results indicate that IT AAV9.trastuzuamb shows promise as a HER2+ BCBM treatment and, more broadly, as a prophylactic measure for patients with HER2+ primary disease to extend survival in the case of BCBM.

(**1**) Kennecke H et al. *J Clin Oncol*. 2010, 28:3271-3277. (**2**) Koo T, Kim I. *Radiat Oncol J*. 2016, 34:1-9. (**3**) Zagouri F, Sergentanis T. *Breast Cancer Res*. 2013, 139:13-22. (**4**) Bousquet, G et al. *J Clin Onc*. 2014, 32:1-5. (**5**) Hinderer C et al. *Mol Ther Methods Clin Dev*. 2014, 1:14051.

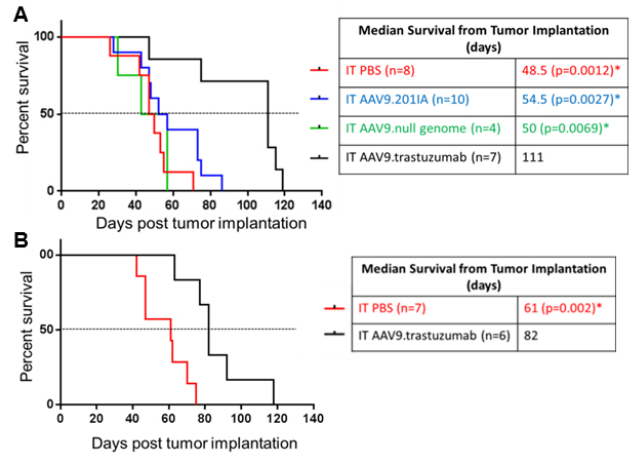

Figure 1. Kaplan-Meyer curves for IT AAV9.trastuzumab prophylaxis (A) and treatment (B) of a Rag1-/- xenograft model of HER2+ BCBM. *Log-Rank (Mantel-Cox) test compared to IT AAV9.trastuzumab treatment.

275. Olfactory Ensheathing Cells for Glioblastoma Gene Therapy

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The olfactory ensheathing cells (OECs) is a unique glial cell type known to play a role in promoting the axonal growth of neuronal receptors from the nasal mucosa towards the olfactory bulb (OB) during physiological turnover. OECs have the ability to migrate from the peripheral nervous system (PNS) to the central nervous system (CNS), which is critical for the development of the olfactory system. During neural regeneration, OECs migrate into the injury site and enhance the axon growth due to the permissive OEC environment. Owing to their ability to myelinate and guide axonal outgrowth, interact with astrocytes, as well as their immuno-modulatory and phagocytic properties, accumulating evidence has shown the potential of OECs in neuronal regenerative medicine, including spinal cord injury and amyotrophic lateral sclerosis in the clinic, but were never studied in the context of cancer. Here, we evaluated for the first time OEC tropism to tumors and their potential use for glioblastoma (GBM) gene therapy, the most malignant type of brain tumors, upon intranasal administration.We extracted/cultured OECs from mice OB

and confirmed their phenotype by immunofluorescence microscopy. We first evaluated in culture using a transwell assay and in vivo in an intracranial GBM model expressing mCherry by instilating 10^5 OECs expressing GFP into the nasal cavity. The intranasal delivery bypasses the blood-brain barrier, limit systemic side effects, and is the natural migration path of OECs to CNS. We observed co-localization of GFP (OEC) and mCherry (GBM cells) both at the primary tumor and the infiltrating single GBM cells showing tropism of OECs to glioma tumors/microenvironment. Knocking down of two cytokines, the tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6), in glioma cells reversed OEC migration indicating that these cytokines are important for OEC tropism to brain tumors. Finally, we evaluated the potential of OEC as a delivery vehicle for brain tumor gene therapy by genetically engineering these cells with a lentivirus vector to express a protein fusion between yeast cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT; CU) under the control of CMV promoter. The CU fusion protein have been shown to have synergistic anti-tumor effect with CD where the 5-FU generated through CD (upon addition of prodrug 5-FC) is further metabolized into 5-FUMP by UPRT. We used a dual secreted luciferase reporter system to monitor cell viability in both OEC (Vargula luciferase; Vluc) and patient-derived GBM stem cells (GSC; Gaussia luciferase; Gluc). The co-culture of these cells in the presence (or not) of 5-FC have shown >80% decreasing in cell viability in GSCs co-cultured with OEC-CU cells (and not the OEC cells control) as well as OEC-CU cells alone, showing that OEC could convert 5-FC to 5-FU, leading to GBM stem cells bystander killing. To corroborate these results in vivo, mice bearing intracranial patient-derived GBM tumors expressing firefly luciferase (Fluc) received intranasally either OEC-CU (or control PBS) and treated with 5-FC (500 mg/kg injected intraperitoneally) for 7 days. We found that mice injected with OEC-CU had a much slower tumor growth and increased in median survival compared to the control group (control, 34 days; OEC-CU, 41 days; n=9/group; $p<0.001$). Our findings show for the first time OECs tropism to glioma tumors and their potential for brain cancer gene therapy using intranasal delivery. OECs have the advantage over other typical stem cells in that they can be easily obtained from the olfactory epithelium and/or OB, a very simple procedure typically done for patients with spinal cord injury allowing autologous transplantation and overcoming ethical issues.

276. CD7-Edited T Cells Expressing a CD7-Specific CAR for the Therapy of T-Cell Malignancies

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Extending the success of CAR T cells to treat T-cell malignancies is problematic since most target antigens are shared between normal and malignant cells, leading to CAR T cell fratricide. CD7 is a transmembrane protein highly expressed in acute T cell leukemia (T-ALL) and in a subset of peripheral T-cell lymphomas. Normal expression of CD7 is largely confined to T- and NK cells, reducing the risk of off-target-organ toxicity. Here, we show that the expression of a CD7-specific CAR impaired expansion of transduced T cells due to residual CD7 expression and the ensuing fratricide. To circumvent this limitation, we used the CRISPR/Cas9 system with CD7-specific single guide RNA to completely disrupt CD7 expression in activated T cells with ~90% efficiency. Loss of CD7 expression did not compromise critical T-cell effector functions, as CD7-knockout ($CD7^{KO}$) T cells expressing a control CD19 CAR demonstrated similar cytotoxicity, cytokine production and expansion as unedited CD19 CAR T cells. Importantly, editing the CD7 gene enabled the expansion (>670 fold after 14 days) of CD7 CAR T cells. The expanded $CD7^{KO}$ CD7 CAR T cells recognized and eliminated T-cell leukemia and lymphoma cell lines and primary T-ALL cells in vitro, resulting in a 90-98% reduction in tumor cell counts after 72h of coculture at an effector-to-target ratio of 1:4. We also observed robust production of IFN-γ and TNF-α by $CD7^{KO}$ CD7 CAR T cells upon coculture with primary T-ALL blasts. A single injection of $2x10^6$ $CD7^{KO}$ CD7 CAR T cells effectively controlled systemic leukemia progression in a mouse xenograft model of aggressive T-ALL and significantly increased median survival of tumor-bearing mice (16 days vs 58 days in mice receiving control vs CAR T cells, P=0.0026). CD7 is also expressed in normal NK- and T-lymphocytes, and we observed profound toxicity of $CD7^{KO}$ CD7 CAR T cells against these subsets in vitro. Therefore, potential T- and NK-cell aplasia could emerge as a potential "on-target off-tumor" toxicity of $CD7^{KO}$ CD7 CAR T cells. However, as our $CD7^{KO}$ T cells are resistant to CD7-directed toxicity and thus could be protective, we tested their ability to combat the most common causes of viremia in highly immunosuppressed patients. We observed that following stimulation with a peptide mix derived from CMV, EBV and adenovirus both $CD7^{KO}$ and control T cells produce equivalent levels of IFN-γ. Moreover, $CD7^{KO}$ CD7 CAR T cells mounted a significant albeit attenuated response to the viral peptide mix, indicating $CD7^{KO}$ T cells with or without CD7 CAR expression could respond to systemic infections and latent virus reactivations. Hence, genomic disruption of CD7 enables the expansion of functional CD7 CAR T cells, and establishes the feasibility of these cells for clinical application in patients with T-cell malignancies. This gene-editing approach additionally may enable the generation of CAR T cells redirected to other T-lineage antigens to broaden the range of targetable tumors.

277. Neural Stem Cell Mediated Oncolytic Virotherapy for Ovarian Cancer

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Introduction. Oncolytic virotherapy is a promising novel cancer treatment that uses replication-competent viruses to induce cancer cell death. While clinical trials are underway for a variety of solid tumors; success has been hampered by rapid immune-mediated clearance/neutralization of the viral vectors, and poor viral distribution to tumor satellites dispersed throughout normal tissue. Neural stem cells (NSCs) are ideal cell carriers that could overcome viral delivery hurdles due

to their intrinsic tumor-tropism and penetration capabilities. Our lab has established a well-characterized, non-immunogenic human NSC line that can selectively distribute to many different solid tumors. Most recently, we observed impressive selectivity and penetration of peritoneal ovarian cancer metastases after intraperitoneal NSC administration. We have engineered our NSCs to produce a conditionally replication-competent adenovirus, CRAd-Survivin-pk7 (CRAd-S-pk7 NSCs). This virus has two notable genetic modifications: (1) a polylysine fiber addition that enables high affinity binding to cell-surface proteoglycans, thus promoting viral entry into the target cell; and (2) a E1A transcriptional modification which prevents viral replication in the absence of the survivin promoter. 80% of ovarian tumors have elevated levels of survivin, which then drives viral replication. Clinical grade equivalent research banks of the CRAd-S-pk7 NSCs cells have demonstrated safety and efficacy in orthotopic glioma models (IND pending), but have not yet been tested in a metastatic ovarian cancer model. We hypothesize that NSCs are able to selectively distribute this virus to ovarian metastases, and provide protection from immune-mediated clearance and neutralization. Our long-term goal is to demonstrate efficacy and safety of CRAd-S-pk7 NSCs for targeted selective tumor killing in patients suffering from stage III ovarian cancer. ***Methods.*** NSG mice with established human peritoneal ovarian (OVCAR8) metastases were injected IP with PBS, Cisplatin, CRAd-S-pk7 NSCs, or a combination of Cisplatin and CRAd-S-pk7 NSCs. Treatment efficacy was determined by tumor burden through bioluminescence imaging. ***Results.*** **T**he combination treatment of CRAd-S-pk7 NSCs and cisplatin resulted in significantly decreased tumor burden (figure 1). *In vitro* data supports *in vivo* results. Studies underway include free virus distribution pharmacokinetic comparisons, and long-term survival.

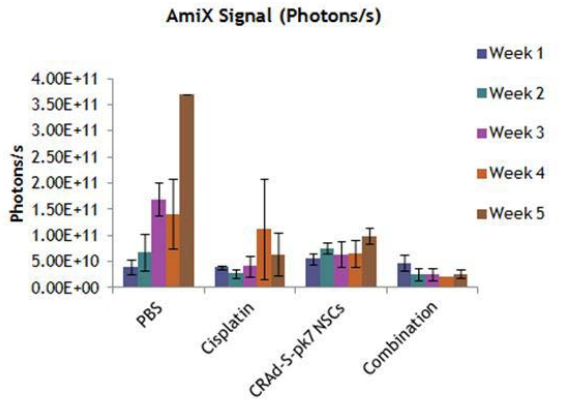

Figure 1. IP-injected CRAd-S-pk7 NSCs demonstrate synergistic effects when combined with cisplatin in a metastatic ovarian cancer model. Signal intensity v. treatment shows mice tumor burden over a 5 week period.

278. Efficacy of a Novel Enzyme/Prodrug Combination for Clostridia Directed Enzyme Prodrug Therapy

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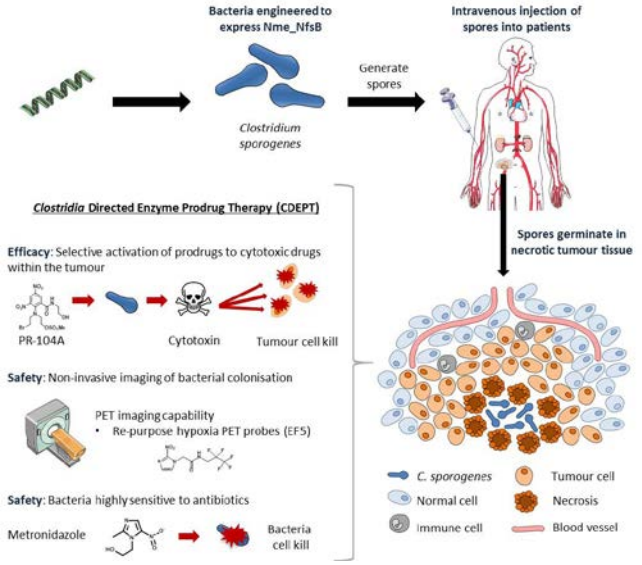
Necrosis is a typical histological feature of solid tumours that is associated with high-risk tumour characteristics and poor patient survival. Necrosis offers the most desirable of attributes for targeted therapy - absolute specificity for neoplasia - being absent from healthy normal tissues. The non-pathogenic anaerobic bacterium *Clostridia sporogenes*, upon injection as spores, germinates selectively in necrotic regions, providing tumour-specific colonisation and offering an opportunity to turn a pathological feature usually associated with treatment failure into a precision therapy. Modest anti-tumour activity of *C. sporogenes* alone encouraged the development of 'armed' vectors (Clostridia Directed Enzyme Prodrug Therapy, CDEPT), but progress was hampered by challenges associated with stable genetic modification of the bacterial genome. This limitation has recently been overcome, making the CDEPT approach clinically feasible for the first time. The prototypical 5-azyridinyl-2,4-dinitrobenzamide CB1954 prodrug for the *E. coli* nitroreductase enzyme NfsB has demonstrated limited utility in clinical trials due, in part, to dose-limiting hepatotoxicity of CB1954 restricting the achievable plasma concentration. To overcome this, a novel nitroreductase (from *Neisseria meningitidis, Nme_NfsB)* has been identified which is able to metabolise PR-104A, a clinical-stage prodrug with increased potency and bystander effect relative to CB1954. We sort to examine the ability of Nme_NfsB to metabolise PR-104A *in vitro* and in an *in vivo* model of CDEPT.

In anti-proliferative assays, human cancer cells expressing Nme_NfsB demonstrated improved sensitivity to PR-104A over CB1954 (WT:Nme_NfsB IC_{50} ratios of 700 and 273-fold respectively). In addition to prodrug activation, we observed Nme_NfsB metabolised other clinically relevant substrates including the 2-nitroimidazole PET imaging agent EF5, suitable for non-invasive monitoring of transgene activity, and the 5-nitroimidazole metronidazole, an antibiotic commonly used to treat *Clostridium* infections, providing an additional safety feature. The optimised gene sequence of Nme_NfsB was then recombined into the *C. sporogenes* genome. The human non-small cell lung cancer xenograft model H1299 was established subcutaneously in NIH-III nude mice and exposed to the vascular disrupting agent vadimezan to induce widespread tumour necrosis. This promoted colonisation by *C. sporogenes* indicating a requirement for necrotic tissue to target germination. Tumour growth delay (TGD) studies found the combination of Nme_NfsB expressing spores and vadimezan produced modest but significant anti-tumour activity compared to untreated controls (TGD = 67%, P=0.004), whilst the inclusion of PR-104 into the treatment schedule provided marked additional activity (TGD = 167%, P<0.001). Successful preclinical evaluation of a transferable gene that metabolises both PET imaging

agents (for vector visualisation) as well as prodrugs (for conditional enhancement of efficacy) is a valuable early step towards the prospect of CDEPT entering clinical evaluation.



Immune Cell Therapies

279. HIV-Specific T Cells from HIV Naive Adult and Cord Donors Target a Range of Novel Viral Epitopes - Implications for a Cure Strategy After Allogeneic Stem Cell Transplant

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Adoptive T cell therapy has been successful in boosting viral-specific immunity post-HSCT, preventing viral rebound of CMV and EBV. However, the therapeutic use of T cells to boost HIV-specific T cell immunity in HIV+ patients has been met with limited success. Despite multiple attempts to eradicate HIV with allogeneic HSCT, there is only one case of functional HIV cure. Hence, we hypothesized that broadly HIV-specific CD8 and CD4 T-cells (HXTCs) could be expanded from patients on ARVs, as well as HIV negative adult and cord blood donors (dHXTC), employing a non-HLA restricted approach for the treatment of HIV+ individuals after autologous or allogeneic HSCT.

We have expanded autologous HXTCs from HIV+ subjects under NCT02208167. To extend this approach to the allo HSCT setting, we generated dHXTCs from HIV naive adults (n=9) and cord blood donors (n=11). IFNg ELISPOT showed dHXTCs from adult donors were specific against Gag, Nef, and Pol (mean=220 IFNg SFC/1e5 cells) versus irrelevant antigen actin (mean=6 SFC/10^5cells) (n=9). Similarly, we are able to produce cord dHXTCs (n=11) that showed specificity to Gag (mean=78 SFC/10^5cells), Nef (mean=96 SFC/10^5cells), or Pol (mean=174 SFC/10^5cells), compared to CTL only control (mean=2 SFC/10^5cells) in IFNg ELISPOT. dHXTCs were polyfunctional producing proinflammatory TNFα, IL2, IL6, IL8, and perforin responses ($p < 0.05$) to HIV stimulation. Importantly, dHXTCs derived from both adult (p=0.0004) and cord blood (p=0.0003) were able to suppress HIV replication compared with nonspecific CD8 T cells when cocultured with autologous CD4 T cells infected with HIV SF162 at an Effector-to-Target ratio of 20:1. Exhaustion marker analysis of cord dHXTC CD3+ cells revealed minimal expression of PD1 (6.8%), TIM3 (3.05%), LAG3 (3.43%), KLRG1 (0.34%), and CD57 (1.08%) (n=6). Comparatively, exhaustion marker analysis of adult dHXTC CD3+ cells revealed higher expression of PD1 (10.13%), TIM3 (7.63%), LAG3 (18.28%), KLRG1 (4.54%), and CD57 (2.43%) (n=6). Epitope mapping of both adult and cord dHXTC products revealed that products contained T cells recognizing unique epitopes not typically identified in HIVpos individuals, which may be critical in overcoming viral immune escape post-HSCT.

In summary, HIV specific T cells can be expanded from HIV+ and HIVneg donors for clinical use. Focusing on donors with HLA types that are associated with well characterized HIV responses (e.g. HLA A02) or associated with delayed progression to AIDS (e.g. HLA B27, B51, B57) may allow us to identify HLA restricted epitopes critical for the successful development of a potent HIV-specific T cell therapeutic. Hence, the administration of dHXTCs derived from naive donors could offer a unique curative strategy post allogeneic stem cell transplant.

280. NKT Cells Expressing a GD2-Specific Chimeric Antigen Receptor with CD28 Endodomain and IL-15 Undergo Dramatic In Vivo Expansion and Mediate Long-Term Tumor Control in a Metastatic Model of Neuroblastoma

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Va24-invariant Natural Killer T cells (NKTs) preferentially localize to the tumor site in neutroblastoma and other types of cancer and have natural antitumor properties that make them attractive as a carrier of tumor-specific chimeric antigen receptors (CARs). Despite effective tumor localization, adoptively transferred NKTs expressing GD2-specific CARs (CAR.GD2) had limited in vivo persistence and transient antitumor activity in a metastatic model of neuroblastoma in NSG mice. In this work, we explored whether expression of IL-15, the main homeostatic cytokine for NKTs, within CAR.GD2 would enhance NKT-cell in vivo persistence and therapeutic efficacy. To that end, we synthesized CAR.GD2 constructs with a costimulatory CD28 or 41BB endodomain with or without IL-15. NKTs that

were transduced with CD28/IL-15 and 41BB/IL-15 CARs secreted similar levels of IL-15 and significantly improved NKT-cell in vitro expansion compared with CD28 and 41BB CARs in response to repeated stimulation with neuroblastoma cells. Three-time weekly stimulation with neuroblastoma cells produced twice as many NKTs with CD28/IL-15 than with 41BB/IL-15 CAR in four independent experiments (P<0.01). After transfer to NSG mice, engrafted with human neuroblastoma xenografts, NKTs expressing IL-15-containing CARs persisted significantly longer compared with those expressing IL-15-less CARs. Only NKTs expressing CD28/IL-15 CAR underwent a progressive in vivo expansion at the site of neuroblastoma metastases, including liver and bone marrow. Indeed, the frequency of CD28/IL-15 CAR NKTs reached 6% of bone marrow cells by day 30 after a single injection and stayed at that level for 3 months. At the same time, human NKTs did not accumulate in normal murine tissues such as skin or intestine and did not induce xeno-GvHD. Treatment with CD28/IL-15 CAR NKTs at day 7 after tumor injection resulted in survival of more than 50% of mice longer than 70 days whereas all mice in other groups had to be sacrificed by day 62 (P<0.001). Thus, a combined use of CD28 costimulatory endodomain and IL-15 in the CAR design enables potent in vivo expansion and anti-tumor activity of CAR.GD2 NKTs cells that could be considered for immunotherapy of neuroblastoma and other solid tumors.

281. Development of KITE-585: A Fully Human Anti-BCMA CAR T-Cell Therapy for the Treatment of Multiple Myeloma

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Background: Multiple myeloma (MM) is a malignancy of plasma cells, with no current curative therapy. One approach to address this unmet need is the use of engineered chimeric antigen receptor (CAR) T cells targeting malignant plasma cells. The efficacy of CD19 CAR T cells targeting hematological malignancies, particularly B-cell leukemias and lymphomas, has been demonstrated in multiple clinical studies including the ZUMA-1 study of axicabtagene ciloleucel (axicel [KTE-C19]) in refractory, aggressive non-Hodgkin's lymphoma (Neelapu, ASH 2016). B cell maturation antigen (BCMA) is ubiquitously expressed on MM cells, plasma cells, and subsets of mature B cells but with limited or absent expression on other tissues. KITE-585 is anti-BCMA CAR T cell immunotherapy. **Methods:** We generated >50 fully human IgGs directed against BCMA using the extracellular domain of the BCMA protein as antigen and selection criteria including affinity, cross-reactivity, and poly-specificity. Following assessment of the binding of the IgGs to a MM cell line known to express BCMA, >10 IgGs were identified that met the criteria for affinity and selectivity and had a >50-fold binding over background. The IgGs that demonstrated the highest specific binding were then sequence-converted to single-chain variable fragments and incorporated into CARs. Additional screening for the specificity of BCMA CAR T cells utilized a cell microarray platform (Retrogenix, Inc) capable of screening approximately 4,500 human plasma membrane proteins (representing up to 75% of the human plasma membrane proteome) individually expressed in human HEK293 cells. **Results:** Human T cells engineered to express these CAR constructs exhibited specific activity against MM cell lines (NCI-H929 and MM.1s). These CAR T cells demonstrated cytolytic efficiencies of >95% at an effector:target (E:T) ratio of 1:1 over a 24-hour period, and had similarly specific killing efficiencies at a very low E:T ratio of 1:10. Antigen-specific production of inflammatory cytokines was observed in response to target cell lines *in vitro* and antigen-dependent proliferation revealed >80% proliferation over a 5-day period for constructs that showed cytolytic activity *in vitro*. Multiple different anti-BCMA CAR constructs that did not display any evidence of tonic signaling and representing distinct epitope binding bins of BCMA were selected for specificity screening. Specific binding of both control mock-transduced and anti-BCMA CAR-transduced T cells were confirmed for different plasma membrane proteins expressed from HEK293 cells. These included known T cell interactors, such as ICOSLG, CD244, and CD86, where binding is proposed to be independent of CAR expression. Subtracting the binding hits of the mock-transduced T cells from the anti-BCMA CAR T cells demonstrated specific binding of CAR T cells to BCMA. In two separate disseminated tumor models of luciferase-labeled NCI-H929 or MM.1s cells injected intravenously (IV), a single IV injection of anti-BCMA CAR T cells significantly increased survival (P<0.01) compared to treatment with control mock-transduced T cells. **Conclusions:** The results of these pre-clinical studies highlight the development of a novel fully human CAR targeting BCMA for the treatment of MM with adoptive transfer of engineered T cells that demonstrate selective and specific binding to BCMA. Phase 1 clinical studies in MM patients with KITE-585 are planned for 2017.

282. Multi-Pathogen-Specific T Cells Effectively Control Invasive Aspergillosis without Inducing Alloreactivity

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Introduction. Adoptive immunotherapy with antigen-specific T cells offers an attractive alternative for the management of viral and fungal infections post allogeneic hematopoietic stem cell transplantation. Towards treating viral and fungal infections by a single T-cell product, we generated multipathogen-specific T cells (mp-STs) that simultaneously target *cytomegalovirus (CMV), Epstein-Barr virus (EBV), BK virus and Aspergillus Fumigatus (Asp)*, from healthy donors, based on a previously developed, rapid and minimally laborious protocol for the production of virus-specific T cells (VSTs), which proved to be clinically safe and effective. Because no single humanized

mouse model exists to reliably recapitulate the condition of infections from multiple pathogens in transplanted hosts, we aimed to test the in vivo functionality and safety of produced mp-STs in a humanized model of invasive aspergillosis (IA). **Methods.** mp-STs were generated by pulsing $1.5x10^7$ mononuclear cells from healthy donors with viral (CMV: IE1, pp65; EBV: EBNA1, LMP2, BZLF1; BK: Large T, VP1) and Asp pepmixes (Crf1, Gel1, SHMT) and culturing them with IL4/7 for 10 days. mp-ST's specificity was assessed by IFN-γ Elispot. A total of $1.5x10^7$ of either immunomagnetically isolated CD3+cells (donor lymphocyte infusions-DLI) or mp-STs were infused in myelo/immuno-ablated NSG mice previously inoculated with Asp conidia intranasally or left uninfected. Mice were evaluated by a 5-parameter sickness score and excised tissues were assessed by histology and immunohistochemistry. **Results.** mp-STs expanded 12-fold, ultimately reaching $23±5x10^7$ cells. All cell lines were polyclonal expressing central and effector memory markers and presented activity against Asp [spot forming cells (SFC)/$2x10^5$cells: 315±82] and viral antigens, when donors were seropositive to targeted viruses [SFC/$2x10^5$cells, CMV: 637±267; EBV: 744±158; BK: 578±118). To first address the safety issue of acute graft-versus-host disease (aGvHD) induction by mp-STs, myelo/immuno-ablated mice were infused with either DLI or mp-STs. DLI-treated mice became sick from day 8 onwards, succumbing by day 20 from clinically and histologically confirmed aGvHD. In contrast, mp-ST-mice survived free of aGvHD until the day of sacrifice (d28). To determine whether mp-STs can provide clinical benefit against IA, conditioned and inoculated with Asp mice, received mp-STs (n=5), DLI (n=4) or left untreated (IA control, n=6). IA- and DLI-groups succumbed to histologically evidenced IA at a median day 6 whereas 3/5 mp-ST-mice survived until sacrifice at day 12 (survival 60%). The day-12 survivors displayed T-cell engraftment in the lung (%$CD3^+$/$CD45^+$: 14±7) and no histological evidence of IA. The two mp-ST-non-survivors died from IA in the absence of T-cell engraftment. Non-specific DLI failed to control IA despite T-cell presence in 3/4 DLI-mice (%$CD3^+$/$CD45^+$ spleen: 58±12, lung: 3±1) which succumbed early, before aGvHD development. **Conclusions.** Overall, engrafted mp-STs effectively controlled IA without inducing alloreactivity. Based on the obtained mp-ST's specificity against all targeted pathogens and the well documented clinical efficacy of multi-VSTs, we expect that our T cell product can become a powerful tool to treat multiple, life-threatening post-transplant infections.

283. Improving CAR T Cell Function by Reversing the Immunosuppressive Tumor Environment of Breast Cancer

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Adoptive transfer of T cells redirected to tumor-associated antigens (TAAs) by expression of chimeric antigen receptors (CARs) can produce tumor responses, even in patients with resistant malignancies. To target breast cancer, we generated T cells expressing a CAR directed to the TAA mucin-1 (MUC1). T cells expressing this CAR (86±1.9%, n=5) specifically killed MUC1-expressing cells (MDA-MB-468 - 45.9±7.3%, MCF-7 - 36.8±3.6) but not MUC1(-) 293T cells (3.7±1.6% specific lysis, 20:1 E:T, n=3). Although these CAR T cells had potent anti-tumor activity against breast cancer cells, when exposed to the Th2-polarizing cytokine IL4 [which is upregulated in tumor samples (Oncomine, $p<0.05$)] we observed a dramatic reduction in their cytolytic potential [IL2 - 45.9±7.3% vs IL4 - 11.3±3.7% specific lysis, 20:1 E:T ratio, n=4]. Thus, to protect our CAR.MUC1 T cells from the negative influences of IL4, we generated an inverted cytokine receptor (ICR) in which the IL4 receptor exodomain was fused to the IL7 receptor endodomain (4/7 ICR). Transgenic expression of this molecule in CAR.MUC1 T cells (55±4.8% double positive cells, n=5), restored the cytolytic function of CAR T cells (30.9±8.1% specific lysis, 20:1 E:T, n=3). Next, to determine the long term effects of this modification we co-cultured transgenic T cells with MUC1+ tumor cells and measured tumor and T cells numbers. In the presence of IL4, only double positive (CAR.MUC1-4/7) T cells expanded and eliminated the tumors in vitro and in vivo. However, upon tumor elimination, transgenic T cells rapidly contracted, demonstrating the antigen- and cytokine-dependence of the product. In conclusion, CAR.MUC1-4/7 T cells can effectively target breast cancer cells and retain their cytotoxic function even in the IL4-rich tumor microenvironment.

284. Immune Modulatory Biomaterials for Cell-Based Therapies

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Immune recognition of implanted biomedical devices initiate a cascade of inflammatory events that result in collagenous encapsulation of implanted materials which leads to device failure. These adverse outcomes emphasize the critical need for biomaterials that do not elicit foreign body responses. One prime example for the use of this technology is with the development of a bioartificial pancreas for the treatment of patients suffering from diabetes. Immunoisolation of insulin producing cells with porous biomaterials provide an immune barrier that is a potentially viable treatment strategy for Type1 diabetic patients. However, clinical implementation has been challenging due to host immune responses to implanted materials. To address this challenge, we have focused our efforts on the development of improved biomaterials for the use in pancreatic islet cell transplantation. To enable the discovery of novel superbiocompatible biomaterials we have developed a high throughput pipeline for the synthesis and evaluation of >1000 material formulations and prototype devices. Here, we describe combinatorial methods we have developed for covalent chemical modification and in vivo evaluation of alginate based hydrogels. Using these methods, we have created and screened the first large library of hydrogels, and identified leads that are able to resist foreign body reactions in both rodents and nonhuman primates. These formulations have been used to generate optimized porous alginate hydrogels fabricated with tuned geometries to enhance biocompatibility. We have identified a lead alginate derivative and capsule formulation geometry that shows minimal recognition by macrophages and other immune cells, and almost no visible fibrous deposition in rodents, and up to at least six months in nonhuman primates. Significantly, our lead formulation has enabled us to achieve

the first long term glycemic correction of a diabetic, immune competent animal model with human embryonic stem-cell derived islet cells, encapsulated using our novel superbiocompatible, chemically modified alginate formulation.

DNA Viruses and Molecular Interventions

285. Potent Antitumor Effect of Tumor Microenvironment-Targeted Oncolytic Adenovirus 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, HmTE and HEmT. Transgene expression driven by each hybrid promoter was markedly higher under hypoxic conditions than normoxic conditions. Moreover, HEmT-driven transgene expression was highly cancer-specific and was superior to that of HmTE-driven expression. A decorin-expressing oncolytic adenovirus (Ad; oHEmT-DCN) replicating under the control of the HEmT 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, oHEmT-DCN exhibited enhanced antitumor efficacy compared with both the clinically approved oncolytic Ad ONYX-015 and its cognate control oncolytic Ad lacking DCN. oHEmT-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 oHEmT-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.

286. Incorporating Synthetic Circuits into Baculovirus Vector for Stringent Control of miRNA and Apoptosis-Inducing Gene Expression in Hepatocellular Carcinoma Cells *In Vitro* and *In Vivo*

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Hepatocellular carcinoma (HCC) is one of the most malignant tumors. MiR-196a is aberrantly overexpressed in young HCC patients yet its roles on HCC tumorigenicity are unknown. Baculovirus (BV) is a promising cancer gene therapy vector thanks to highly efficient transduction of various mammalian cells including hepatocytes, but in vivo delivery of BV may result in undesired gene expression in normal tissues and cells. Here we constructed a baculovirus expressing miR-196a sponge for miR-196a knockdown in HCC cells, which hindered sphere formation and angiogenic factor secretion, suggesting that miR-196a contributes to HCC malignancy. We further unraveled that miR-126 was barely expressed in HCC cells but highly expressed in normal cells. To restrict baculovirus-mediated apoptotic gene expression in HCC cells but not in normal cells, we developed a synthetic circuit-based baculovirus system for transcriptional targeting which was controlled by cellular miRNA and RNA-binding protein (L7Ae) wired to operate switches with high stringency. The baculovirus Bac-hBax-Circuit contained 2 expression cassettes: one expressing L7Ae with miR-196a binding sites at the its 3'-UTR while the other expressing the apoptosis-inducing gene hBax with K-turn motif at 5'-UTR and miR-126 binding sites at the 3'-UTR. Transduction of normal cells (abundant in miR-126 but low miR-196a levels) resulted in shutdown of hBax due to miR-126 binding to the 3'-UTR of hBax and the expressed L7Ae binding to the K-turn motif. Conversely, transduction of HCC cells abundant in miR-196a but scarce in miR-126a led to simultaneous downregulation of miR-196a and L7Ae, which allowed for hBax expression owing to the low miR-126 expression and hence killing of HCC cells. Furthermore, portal vein injection of the circuit-based BV into orthotopic HCC mice models hindered tumor growth and triggered HCC cell death, without harming adjacent normal cells. This study demonstrated the potential of incorporating a synthetic circuit into baculovirus vector as a novel approach to targeted HCC therapy.

287. Retargeted oHSV Vector Development for Breast Cancer Treatment

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Oncolytic herpes simplex viruses (oHSV) offer several advantages for the treatment of cancer. HSV is a non-integrating viral vector that can be designed to infect and replicate in specific tumor cell types and has a large carrying capacity, permitting the incorporation of immune-modulatory transgenes to further assist in tumor cell killing. In the current study, we have designed a fully retargeted oHSV that can preferentially infect and lyse breast cancer cells. Strategies for full retargeting of HSV infection require virus detargeting from its cognate receptors (HVEM and nectin1), recognized by the virus attachment/entry component glycoprotein D (gD), and introduction of a new ligand into gD that allows entry through recognition of the corresponding cellular receptors. One unique target, the GFRα1 receptor, displays a limited expression profile in normal adult tissue, but is upregulated in a subset of breast cancers. To target an HSV vector for entry exclusively into cells expressing the GFRα1 receptor we employed its ligand, GDNF. We replaced the signal peptide and HVEM binding domain of gD with pre-pro-(pp)GDNF to create a GFRα1 targeting protein, gD(Y38)_GDNF, that can still bind nectin1. Virus expressing gD(Y38)_GDNF was propagated on cells expressing nectin1 and purified virus was shown to enter nectin1-deficient J1.1-2 and B78H1 cells in a GFRα1-dependent manner. U2OS cells engineered to express GFRα1

demonstrated robust virus entry and spread, allowing us to create and propagate a fully retargeted virus that can no longer bind nectin1, gD(Δ38)_GDNF. This fully retargeted virus was shown to enter and spread in GFRα1-positive breast cancer cells, resulting in significant, MOI-dependent tumor cell death *in vitro*. We are currently testing this oHSV for effective tumor treatment in an *in vivo* breast cancer mouse model system. Retargeted viruses that do no enter normal tissues, but specifically enter and lyse tumor cells offer a distinct advantage for the safety profile of oHSV and may allow systemic treatment.

288. Capsid-Modified AdC7 Vector Expressing *Pseudomonas aeruginosa* OprF for Therapeutic Immunization Against Chronic Pulmonary *P. aeruginosa* Infection

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Respiratory infections caused by *Pseudomonas aeruginosa* are a major clinical problem, particularly in patients with cystic fibrosis (CF) and other chronic lung diseases associated with bronchiectasis. There is currently no vaccine against *P. aeruginosa*. A post-exposure vaccine that eliminates already established *P. aeruginosa* from the respiratory tract, could be useful in the management of chronic *P. aeruginosa* colonization. Replication-deficient adenoviral (Ad) vectors based on non-human serotypes, to circumvent the problem of pre-existing anti-Ad immunity in humans, are an attractive platform for vaccine against *P. aeruginosa*. We have previously found that a non-human primate-based AdC7 vector expressing outer membrane protein F (OprF) of *P. aeruginosa* (AdC7OprF) was more potent in inducing lung mucosal and protective immunity compared to a human Ad5-based vector. In addition, genetic modification of the AdC7 fiber to display an integrin-binding arginine-glycine-aspartic acid (RGD) sequence can further enhance mucosal protective immunogenicity of AdC7OprF. In this study we investigated the efficacy of post-exposure vaccination by AdC7OprF.RGD to clear the already established *P. aeruginosa* in wild-type or CF mice and rat models. Intratracheal inoculation of *P. aeruginosa* (clinical strain RP73) encapsulated in agar beads was used to establish persistent infection. Intranasal immunization of *P. aeruginosa* infected wild-type mice with AdC7OprF.RGD (10^10 pu/mouse) induced significantly high anti-OprF serum IgG and mucosal IgA antibodies as early as 1 week of immunization that further increased to higher levels after 2 week of immunization compared to AdC7Null or PBS inoculated mice ($p<0.05$; all comparisons). In addition to robust humoral response, immunization with AdC7OprF.RGD induced OprF-specific IFN-γ and IL-4 T-cell responses ($p<0.05$; all comparisons). Importantly, the AdC7OprF.RGD immunized mice showed higher clearance of *P. aeruginosa* from the infected lungs after 1 week or 2 weeks of immunization ($p<0.05$; all comparisons). Next we evaluated the efficacy of AdC7OprF.RGD immunization in CFTR-deficient CF mice (C57BL/6 Cftr^{tm1unc}). Clearance of *P. aeruginosa* was delayed in the CF mice compared to WT mice ($p<0.05$). Intranasal immunization of *P. aeruginosa* infected CF mice with AdC7OprF.RGD (10^10 pu/mouse) elicited higher anti-OprF serum IgG compared to AdC7Null inoculated mice ($p<0.05$). Likewise, *P. aeruginosa* titers were decreased in the CF mice after 10 days of immunization ($p<0.05$). Similarly, intranasal immunization of *P. aeruginosa*-infected Sprague Dawley rats with AdC7OprF.RGD (5x10^10 pu/rat) induced protective anti-*P. aeruginosa* immunity that lead to decreased lung *P. aeruginosa* titers compared to AdC7Null control ($p<0.05$). These data suggest that immunization with AdC7OprF.RGD is effective in clearing existing colonization with *P. aeruginosa* and could thus be a useful vaccine platform in cystic fibrosis.

289. A Novel Prime-Boost Ebola Virus Vaccine Study Based on Recombinant Chimpanzee Adenovirus Vector

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Ebola virus disease is one of the most contagious and dangerous disease that causes the extremely high morbidity and mortality. The recent outbreak of ebola hemorrhagic fever in several West African countries during 2014 to 2016 leads to the mortality rate as high as 39.5%, which poses a serious public health problem. Here we show a prime-boost ebola virus vaccine strategy based on two novel recombinant replication-deficient chimpanzee adenoviruses (Ad) vectors AdC7 and AdC68 expressing the ebola virus (EBOV) surface glycoprotein (GP) identified in the 2014 outbreak. A lot of previous studies were performed with Ad vectors based on human serotype 5 (AdHu5). Compared to AdHu5, the chimpanzee Ad exhibit much lower seroprevalence in human beings, which made them great alternative Ad vaccine vectors. We utilized a murine intramuscular injection model with AdC7-EBOVgp prime and AdC68-EBOVgp boost 4 weeks later. The humoral and cellular immune responses against EBOV GP were determined by a EBOV GP specifc enzyme-linked immunosorbent assay, a EBOV pseudotyped-based neutralization antibody assay, and a flow cytometry assay for intracellular cytokine detection. We found that the AdC7 prime and AdC68 boost elicited much stronger and longer B-cell and T-cell immune responses to EBOV than those induced by the AdC7 alone. This novel vaccine regimen could serve as promising long-term immune protection vaccine candidate against ebola virus infection.

290. Highly Efficient Sendai Virus Mediated CRISPR/CAS9 Gene Editing in Hematopoietic Stem Cells

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A highly efficient CRISPR/Cas9 delivery system is critical for hematopoietic stem cell (HSC) based gene therapy for many diseases. Current, non-viral and viral methods (lentivirus and adeno-associated virus serotype 6) in common use for delivery of CRISPR/Cas9 have several drawbacks including limited efficiencies in HSCs. Persistence of DNA based viral vectors may also lead to greater off-target effects

in the long-term. In previous work, we repurposed Sendai virus (SeV), as a delivery system for highly efficient Cas9-mediated gene editing (75-98% without selection) in human cells, including primary human macrophages, with minimal off-target effects. SeV is non-pathogenic in humans, has an established safety record, and has been extensively studied and modified for gene therapy applications. SeV is an RNA virus that replicates solely in the cytoplasm and has no viral DNA phase, thereby eliminating any potential for undesired random integration into the host genome. Flanking the guide RNA with self-cleaving ribozymes allowed this RNA vector to produce the precise ends required for gRNA function, and we showed for the first time that self-cleaving ribozymes were tolerated *within* the paramyxovirus genome.

Here, we show further safety improvements in our SeV CRISPR/Cas9 delivery system by introducing mutations to confer temperature sensitivity (*ts*) that allows removal of the virus at non-permissive temperatures (37-38°C) once editing at a permissive temperature (34°C) has occurred. This non-cytopathic *ts* rSeV-Cas9 vector transduced human fetal liver and peripheral blood mobilized CD34+ HSCs at ~90% and resulted in ~80% mutagenesis of the targeted locus (ccr5) within 2 days. Shifting to 37°C resulted in rapid loss of the rSeV-Cas9 vector. Introducing *ts* mutations into replication-deficient SeV vectors (e.g. by removing the fusion protein essential for virus entry) will likely synergistically enhance the safety of our SeV-Cas9 vectors for clinical applications requiring highly efficient gene editing in HSCs. The latest Cas9 modifications such as chimeric fusions for base-editing or mutations that confer increased specificity can be easily incorporated into our versatile SeV-Cas9 vector. Additional applications of our rSeV-Cas9 delivery system will be discussed.

Clinical Trials for Neurologic and Neurosensory Disorders

291. 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 is a devastating, monogenic neurodegenerative disease. Children with its most severe form, SMA Type 1 (SMA1), will never sit unassisted, roll over or maintain head control. A natural history study of SMA1 children reported that none achieved a CHOP-INTEND score of more than 40 by 6 months of age (one transient exception at ~41) and 75% died or required permanent ventilation by 13.6 months. This trial explores safety and efficacy of a single intravenous administration of gene therapy in SMA1. This is the first gene therapy (AVXS-101) trial in SMA1, a rapidly lethal neurologic disease. AVXS-101 delivers the SMN gene in a single intravenous dose via the AAV9 viral vector, which crosses the blood-brain barrier. In this ongoing 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 6.7e13vg/kg (Cohort 1, n=3) or 2.0e14vg/kg (Cohort 2, n=12). The primary objective is safety and the secondary objective is survival (avoidance of death/permanent ventilation). CHOP-INTEND scores and motor milestones are also evaluated. AVXS-101 appeared to have a favorable safety profile, to be generally well tolerated, and to improve survival (September 15, 2016 data cut-off). All patients were alive at data cut-off and only 1 patient, from Cohort 1 (low-dose cohort), required permanent ventilation at 28.8 months of age. All patients reaching 13.6 months did so free of permanent ventilation. Patients in Cohort 2 (proposed therapeutic dose cohort) were free of permanent ventilation and demonstrated improvements in motor function: 11/12 had achieved CHOP-INTEND scores ≥40 points, 11/12 achieved head control and sat with support, and 8/12 sat unassisted. Two patients could crawl, stand and walk independently. In contrast with the natural history of the disease, a single intravenous administration of AVXS-101 appears to demonstrate a positive impact on the survival of both cohorts and a dramatic, sustained impact on motor function in Cohort 2: 11/12 patients achieved CHOP-INTEND scores and motor milestones rarely or never seen in this population. A clinical update will be given at the time of presentation.

292. A Phase 1/2 Clinical Trial of Systemic Gene Transfer of scAAV9.U1a.hSGSH for MPS IIIA: Safety, Tolerability, and Preliminary Evidence of Biopotency

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Sanfilippo syndrome type A (mucopolysaccharidosis type IIIA), a lysosomal storage disorder due to mutations in the *SGSH* 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 recombinant scAAV9.U1A.h*SGSH* 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, blood leukocyte SGSH 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). Three low-dose (5 X 10^{12} vg/kg) subjects (ages 6.4, 6.9, and 5.3 years) and one high-dose (1 X 10^{13} vg/kg) subject (age 2.5 years) have tolerated gene transfer well without significant adverse events, and enrollment in the high-dose cohort is continuing. All subjects received oral prednisolone from Day -1 through at least Day 60; 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. In comparison to the screening exam, abdominal MRI in the low-dose cohort showed a decrease in liver volume by a mean of 17.7% (range, 13.9 - 20.5%) and in spleen volume by 17.6% (range, 5.2 - 29.8%) at Day 30 post-injection. In the two patients evaluated at 6 months post-injection, this effect was sustained, with a liver volume further decreased by 29.7 - 30.3% and spleen volume by 2.2 - 12.9% from baseline. There is a decrease in urinary (mean decrease of 57.6%, range 44.4-72.7%) and CSF (mean decrease of 25.8%, range 25-27.3%) HS fragments at Day 30, with the latter suggesting that systemic administration of the vector resulted in successful gene transfer across the blood-brain barrier. The two subjects assessed at the 6-month timepoint showed evidence for stabilization of scores in several Mullen subdomains (in 4 of 5 in the first and in 2 of 5 in the second subject). Adaptive behavior ratings on the Vineland also stabilized. Both subjects showed improved ability to complete individual items on the Leiter-R non-verbal IQ assessment resulting in improved raw scores, although standard score changes could not be measured as both subjects performed at the floor level of the assessment. In aggregate these data demonstrate safety and tolerability of the vector, and indicate biological activity in multiple tissues including the liver, spleen, and the CNS. Suggestions of a stabilizing effect on adaptive behavior and cognitive function, although promising, require confirmation from additional subjects and future assessments to be conducted at the predefined 12 month time point. This study is supported by Abeona Therapeutics.

293. Does a One Time Retinal Gene Therapy Last Long: A Question Answered by the Brain

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Introduction: A one-time administration of retinal gene therapy (GT) has been reported to be an effective treatment for patients with Leber's Congenital Amaurosis caused by an RPE65 mutation (LCA2). However the long-lasting benefit of GT in this condition has been a controversial issue. A recent study presented clinical evidence to the efficacy and longevity of this treatment that was largely based on patients' clinical follow-up outcome measures. The goal of this study is to independently seek an answer to this question by interrogating longitudinal brain responses to retinal GT using functional magnetic resonance imaging (fMRI). To further support the results from longitudinal brain response to GT, correlations between the fMRI results and patients' clinical measures were evaluated.

Methods: Seven REP65 subjects participated in the longitudinal neuroimaging study. In a Phase I LCA2 clinical trial subjects received GT to their worst seeing eye. In a follow-on (FO) study to Phase I clinical trial, GT was administered to the contralateral eye. fMRI was longitudinally performed before and 1-3 years after FO GT. Subjects were presented with three levels (high, medium, and low) of a full-field contrast-reversing checkerboard stimuli via MRI-compatible video goggles. MRI scans were performed on a 3T research system at CHOP using a 32-channel head coil.

Results: Baseline fMRI showed minimal to no cortical activations in response to visual stimulation. Longitudinal fMRI for 1-3 years after retinal GT showed significant increases in cortical responses for all subjects, with increased activation persisting up to three years after GT. Repeated measures analysis showed significant associations between the magnitude of cortical activations and patients' clinical measures such as full field light sensitivity threshold (FST) for white, red, and blue colors, visual field (VF), and pupillary light reflex (PLR).

Conclusions: Consistent with our previous study, fMRI result from the FO clinical trial (subretinal injection of the contralateral eye) displayed intact visual pathways that became responsive and strengthened after FO GT administration for a group of LCA2 patients. fMRI results suggest a lasting impact for at least three years for a one-time retinal GT. These neuroimaging results are also consistent with recently reported longitudinal clinical outcome measures and significantly correlate with patients' clinical outcomes. One of the important consequences of this work is the introduction to a new potential way to objectively quantify the visual benefits to sight-restoring therapy for a variety of treatments, including gene and cell-based therapies.

294. Postmortem Assessment of Vector Biodistribution in the First-in-Human Intrathecal scAAV9 Gene Therapy Trial for Giant Axonal Neuropathy

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Giant axonal neuropathy (GAN) is a rare pediatric neurodegenerative disorder characterized by progressive sensory and motor neuropathy that presents as early as 2-3 years of age and with mortality during the second or third decade of life. GAN is caused by autosomal recessive loss-of-function mutations in the *GAN* gene that encodes for the gigaxonin protein. Gigaxonin plays a role in the organization/degradation of intermediate filaments (IFs), and GAN is pathologically characterized by large axonal swellings filled with disorganized aggregates of IFs. While GAN is primarily described as a progressive peripheral neuropathy, diffuse pathology from disorganized IFs is apparent throughout the entire nervous system and other organ systems. An NIH-sponsored Phase I study (NCT02362438) is underway to test the safety of intrathecal (IT) administration of scAAV9/JeT-GAN to treat the most severe aspects of GAN, namely the motor and sensory neuropathy. Besides preclinical efficacy studies, the safety and biodistribution of scAAV9/JeT-GAN was investigated in mice, rats, and non-human primates (NHPs) that received a single IT overdose of scAAV9/JeT-GAN. Gigaxonin gene transfer is the first proposed therapy for GAN, and to our knowledge this is the first IT delivery of a gene therapy vector in humans. One patient in the trial, who received a single lumbar IT injection of $3.5x10^{13}$ vg and who clinically was doing well in follow up after gene transfer, died unexpectedly at 8 months post injection of a cause that was reviewed and deemed unrelated to treatment with scAAV9/JeT-GAN. The family provided consent for an autopsy to include use of tissue for research, allowing specimens to be evaluated for vector DNA biodistribution and long-term transgene (mRNA) expression by quantitative PCR. In total, the results were largely consistent with preclinical studies in mice, rats, and NHPs. The vector DNA was present at higher concentrations in the lower spinal cord at up to 0.5 transgene copies per diploid host genome, and vector DNA was further detected in all CNS tissues as well as peripheral tissues, at varying lower levels. Vector DNA was present in all dorsal root ganglia examined, however, the concentration of vector DNA was lower than we have previously found in rodents and NHPs. Gigaxonin transgene mRNA expression of the codon-optimized human GAN transgene was detectable across the CNS and select peripheral tissues, albeit at very low levels. This was as expected, due to the use of the weak synthetic JeT promoter. Two important conclusions can be drawn: 1) The AAV9 vector biodistribution patterns seen in mice, rats, and NHPs translated faithfully to this single human subject. 2) The vector design and approach was able to confer long-term transgene expression, also consistent with the levels seen in preclinical models. In whole, our findings suggest that biodistribution studies in preclinical rodent and NHP models accurately model the human biodistribution of AAV9 after intrathecal administration.

295. Gene Therapy for Aromatic L-Amino Acid Decarboxylase Deficiency: 5 Years After AAV2-hAADC Transduction

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Deficiency of aromatic L-amino acid decarboxylase (AADC) activity impairs the production of monoamine neurotransmitters dopamine, serotonin, epinephrine, and norepinephrine. In severe forms of AADC deficiency, neither dopamine agonists nor other medications effectively relieve patients' symptoms. We have injected an AAV2-hAADC vector, named as AGIL-AADC currently, into bilateral putamens of patients with AADC deficiency, and demonstrated improvements in patients' motor function. We now present data from 5 patients who have been followed for more than 5 years after treatment. These patients did not have head control and had not achieved other major motor milestones prior to gene transduction, but started to gain new motor skills after gene transduction. Motor development and cognitive function showed improvement over this 5-year period, with the most substantial gains observed during the first two years after gene transduction. At 5 years after gene transduction, FDOPA PET still exhibited signals of AADC activity over the putamens. Patients' anti-AAV2 antibody titers rose after gene transduction, peaked a few months later, and then decreased. There were no signs of cerebral or systemic immune reaction during the follow up period. Therefore, treatment with AAV2-hAADC demonstrates encouraging preliminary evidence of long-term safety and therapeutic efficacy for patients with AADC deficiency. Recently, another phase I/II trial has completed, and a phase IIb trial with dosage escalation is currently enrolling patients.

296. Update on the First-in-Human Clinical Study Evaluating Neural Stem Cells in Patients with Parkinson's Disease

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Parkinson's disease (PD) is a chronic and progressive movement disorder characterized by the degeneration of dopamine (DA) neurons in the substantia nigra. There is currently no cure for PD and available therapies do not stop disease progression. Cell based therapies have shown considerable promise in some PD patients because they can achieve significant biochemical and clinical improvements decades after implantation. We have demonstrated in preclinical PD models that intracranial transplantation of human parthenogenetic derived neural stem cells (ISC-hpNSC®) is safe and promotes symptomatic relief by increasing DA levels and DA neuron innervation. We received clinical trial approval from the Australian Therapeutic Goods Administration (TGA) and are currently conducting a First-In-Human study to evaluate the safety and functional activity of ISC-hpNSC® in PD patients (ClinicalTrials.gov: NCT02452723). This is a single-arm, open-label, Phase I study evaluating three dose regimens of 30, 50 or 70 million ISC-hpNSC® in 12 patients divided into three cohorts of four. The patients receive stereotactic bilateral injections into the caudate nucleus, putamen and substantia nigra. Following transplantation, patients are monitored for 12 months with an additional 5 year long-term follow-up. PET scans and neurological assessments including Unified Parkinson Disease Rating Scale (UPDRS), Hoehn and Yahr and other rating scales are performed at screening and at specified intervals post-transplantation. Clinical responses are compared to baseline to determine preliminary efficacy. Interim data of patients transplanted with 30 million ISC-hpNSC® shows that there are no test article related serious adverse events. No complications, including dyskinesia, or safety issues associated with the administration procedure or immunosuppressive regimen have been reported and preliminary efficacy data will also be presented. In summary, ISC-hpNSC® is a promising novel therapeutic candidate for the treatment of PD.

Pharmacology, Toxicology and Assay Development

297. Pharmacology and Toxicology Studies Conducted for the First-in-Human Clinical Study of Neural Stem Cells in Parkinson's Disease

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Cell-based therapies hold great promise in the treatment of Parkinson's disease (PD). Grafted fetal tissue has shown significant long-term biochemical and motor improvements in some PD patients. Unfortunately the source of fetal tissue is limited, ethically controversial and clinically impractical and alternative sources are needed. Human parthenogenetic stem cells (hpSCs) offer a more practical alternative because they are pluripotent stem cells that can be expanded indefinitely *in vitro* and generate an unlimited supply of neural tissue for transplantation. Unlike human embryonic stem cells, hpSCs are derived from unfertilized oocytes, avoiding the ethical controversy associated with the destruction of a potentially viable human embryo. We have demonstrated that intracranial transplantation of hpSC-derived neural stem cells (ISC-hpNSC®) into the striatum and substantia nigra promotes repair of the nigrostriatal system and increases dopamine (DA) levels in proof-of-concept preclinical studies. We have then manufactured master and working cell banks of ISC-hpNSC® under cGMP and conducted a series of preclinical studies. Here we present an overview of the pharmacology and toxicology studies conducted for the approval of a first-in-human clinical study. We conducted *in vitro* experiments and demonstrated the absence of residual pluripotent hpSC in the final clinical grade formulation of ISC-hpNSC®. We conducted an acute toxicity study determine the maximum feasible dose of ISC-hpNSC® that can be safely administered to athymic nude rats. We performed a long-term tumorigenicity and biodistribution study in 300 athymic nude rats and discovered that the tumorigenic potential of ISC-hpNSC® is negligible. Finally, we conducted a 12 month pharmacology and toxicology study in MPTP-lesioned non-human primates (NHP) with moderate to severe PD. We observed that ISC-hpNSC® promoted behavioral recovery and increased striatal DA levels and DA neuron innervation in NHP without signs of toxicity, ectopic tissue formation, tumors, or biodistribution to other organs. Overall, results of these preclinical studies showed that administration of ISC-hpNSC® is safe and effective and allowed for the approval of the world's first pluripotent stem cell based therapy for PD (ClinicalTrials.gov: NCT02452723).

298. Insertion Site Analysis as a Component of Gene Therapy Product Characterization

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Integration of new DNA into a genome is a required step in the retroviral replication cycle, and the ability to integrate new DNA precisely has been adapted for therapeutic gene transfer. Data on the locations of integration sites generated during gene therapy can help to track corrected cell populations, detect potential clonal expansions of cells, and identify integration-associated adverse events. Our laboratory has developed a comprehensive pipeline for integration site analysis (INSPIIRD, integration site pipeline for paired-end reads) and statistical tools for data visualization and interpretation. Initially, genomic DNA is randomly fragmented through sonication and amplified through a linker-mediated nested-PCR. This method samples from the complete genome in an unbiased manner, generates data supporting abundance calculations, and controls production of artifacts. We tested the pipeline with multiple simulated and synthetic data sets to optimize processing parameters based on the comparison to truth. A database was designed to house data generated by the Illumina platforms and which supports tools for statistical analysis and data reporting. Integration site positions are compared to a curated lists of cancer-associated genes for interrogation of possible vector driving of cell proliferation or adverse events. Data outputs range from interactive browsers to heat maps summarizing integration site placements near genomic features to detailed patient reports summarizing longitudinal data for clinical monitoring. As examples of output, we will present data from recent trials in two areas: 1) stem cell gene therapy to treat inherited immunodeficiencies and 2) T-cell therapy using chimeric antigen receptors delivered by lentiviral vectors to treat CD19-positive leukemias.

299. Evaluating Safety and Efficacy of the AAV2tYF-PR1.7-CNGA3 Vector in CNGA3-Deficient Sheep

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Purpose: Achromatopsia is an inherited retinal disorder characterized by markedly reduced visual acuity, extreme light sensitivity and absence of color discrimination. AGTC is developing a recombinant adeno-associated virus (AAV) vector expressing human cyclic nucleotide-gated channel alpha subunit (AAV2tYF-PR1.7-CNGA3) for treatment of CNGA3-related achromatopsia. Here we report results of a toxicology and efficacy study of this vector administered by subretinal injection in CNGA3-deficient sheep. **Methods:** A 0.5 mL subretinal injection of AAV2tYF-PR1.7-CNGA3 or AAV5-PR2.1-hCNGA3 (a vector previously shown to rescue cone photoreceptor responses), was given to groups of 4 or 5 animals in the right eye at one of two dose levels. The left eye received a 0.5 mL subretinal injection of vehicle (4 animals) or was untreated (9 animals). Toxicity assessment was based on mortality, clinical observations, ophthalmic examinations, electroretinogram (ERG), and clinical and anatomic pathology. CNGA3 expression was assessed by immunohistochemistry. Efficacy was assessed by cone ERG responses and maze navigation testing performed before, and then 6 and 12 weeks after treatment. **Results:** No systemic toxicity was associated with treatment. Most animals had mild to moderate conjunctival hyperemia, chemosis and subconjunctival hemorrhage immediately after surgery that generally resolved by post-operative Day 7. No clear and consistent test article-related effects were noted in any group during the 12 week study. Two animals treated with the higher dose of AAV2tYF-PR1.7-CNGA3 had microscopic findings of outer retinal atrophy, with or without inflammatory cells in the retina and choroid that were procedural- and/or test article-related. All vector-treated eyes developed cone-mediated ERG responses with no change in rod-mediated ERG responses. Behavioral maze testing showed significantly improved navigation times and reduced numbers of obstacle collisions in all vector-treated eyes compared to their control eye or pre-dose results in the treated eye. Conclusions: Subretinal injection of AAV2tYF-PR1.7-CNGA3 in CNGA3-deficient sheep was well tolerated with no clinically important toxicology findings. Cone-mediated ERG responses as well as photopic behavior were rescued in all vector-treated eyes. These results support the use of AAV2tYF-PR1.7-CNGA3 in clinical studies in patients with achromatopsia caused by mutations in CNGA3. A Phase 1/2 clinical trial evaluating AAV2tYF-PR1.7-hCNGA3 is scheduled to begin in 2017.

300. Nonclinical Safety Evaluation of scAAV8-hRLBP1 (CPK850) for Treatment of RLBP1-Associated Retinitis Pigmentosa

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Retinitis pigmentosa is a form of retinal degeneration usually caused by genetic mutations in key functional proteins. We have previously demonstrated efficacy in a mouse model of RLBP1 deficiency with a self complimentary AAV8 vector carrying the human gene for wild-type RLBP1 under control of a short RLBP1 promoter (Choi et al 2015), now referred to as CPK850. In this communication, we describe the nonclinical safety profile of this construct when dosed into the subretinal space. We conducted a rodent study comparing the effects in Rlbp1+/+ and Rlbp1-/- mice three months after dosing and a non-human primate study with an interim sacrifice at three months and a terminal sacrifice at six months after dosing. Multiple dose levels of the vector ranging from $3.2x10^7$ vg/ul to $3.3x10^9$ vg/ul were used. In mice, OCT and histopathological analysis indicated retinal thinning that appeared to be dose-dependent for both Rlbp1 genotypes with no qualitative difference noted between wild-type and Rlbp1-/- mice. In the nonhuman primate study, dose dependent intraocular inflammation and retinal thinning was observed, with the inflammation resolving slowly over time. Pre-existing anti-AAV8 antibodies were detected in 78% of the animals and the presence of pre-existing ADA did not appear to correlate with ocular inflammation or expression of the RLBP1 gene. At the three month timepoint, a subset of animals from each group were dosed in the contralateral eye with $3.3x10^7$ vg/ul. No new observations were noted compared to eyes initially dosed at that level, in spite of the generation of treatment-boosted or treatment-emergent anti-AAV8 antibodies. Dose-dependent ocular RLBP1 expression was observed at the three-month time point in satellite animals of the non-human primate study. Biodistribution analysis was performed in a dedicated three-month rat study as well as from satellite animals in the nonhuman primate study. The vector was largely detected in ocular tissues as well as at low levels in the optic nerve, superior colliculus and lateral geniculate nucleus with limited distribution outside of these tissues. These data suggest that a dose of ~$3x10^7$ vg/ul led to no adverse events beyond those attributed to the injection procedure and the findings at higher doses are consistent with other AAV-based therapies currently in the clinic.

301. Pre-Clinical Development of a Genetically-Modified Human Dermal Fibroblast (FCX-007) for the Treatment of Recessive Dystrophic Epidermolysis Bullosa (RDEB)

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Recessive dystrophic epidermolysis bullosa (RDEB) is an autosomal recessive, inherited skin disease caused by null mutations within the type VII collagen gene (*COL7A1*). The mutations cause an absence or reduction of functional type VII collagen (COL7), which incorporate into anchoring fibrils that maintain binding of the epidermis to the dermis. The disease is characterized by mechanical fragility and repeated blister formation in the sub-lamina densa, at the level of the structurally defective anchoring fibrils. Currently, there is no effective therapy for this disease, and death is usually the result of aggressive squamous cell carcinoma, sepsis, or malnutrition. We are developing an autologous, genetically-modified fibroblast cell therapy that is anticipated to improve skin function in RDEB patients through restoration of type VII collagen levels. A patient's fibroblasts are harvested, genetically modified *ex-vivo* with a functional *COL7A1* gene, and expanded in culture (FCX-007). *Ex vivo* transduction occurs through the use of a replication-defective, self-inactivating (SIN) lentiviral vector. After expansion, the fibroblasts are administered back to the patient as local intradermal injections into target wound margins. The resulting increase in type VII collagen and anchoring fibrils is anticipated to stabilize the connection between skin layers, reduce blistering and promote wound healing. *In vitro* product development data indicates that cGMP scale FCX-007 cells express full-length type VII collagen exhibiting the proper trimeric form, size and binding functionality. The integrated transgene copy number and COL7 expression is dependent on viral dose. We also evaluated FCX-007 using pre-clinical animal models by implanting *in vitro* cultured human RDEB patient and normal human skin xenografts onto immunodeficient SCID mice. Following intradermal injection of FCX-007 cells into RDEB patient xenografts, we noted *in vivo* localization of human type VII collagen into the dermal-epidermal basement membrane without any overt signs of toxicity or tumorigenicity. Additionally, FCX-007 injected in normal skin of SCID mice was assessed in 1, 3 and 6 month toxicology studies with no toxicity or tumorigenicity observed. The data presented support an Investigational New Drug (IND) filing. We also present the study design of an ongoing Phase I/II clinical trial to treat RDEB subjects with FCX-007. The endpoints for the study are to evaluate safety (primary), efficacy and durability (secondary).

302. Prenylation of Rab6a as a Potency Assay for Choroideremia Gene Therapy

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Background Choroideremia (CHM) is a rare, X-linked recessive retinal dystrophy caused by mutations in the *CHM* gene. This gene encodes for Rab escort protein 1 (REP1), which is ubiquitously expressed in human cells and plays a key role in intracellular trafficking through the prenylation of RabGTPases. Deficiency of REP1 prevents Rab proteins of being prenylated and delivered to the target membrane, causing cellular dysfunction and ultimately cell death. As the use of adeno-associated viral (AAV) vectors for choroideremia gene therapy becomes a clinical reality, there is a need for reliable and sensitive assays to determine the activity of exogenously delivered *CHM*. *In vitro* prenylation using a biotinylated lipid donor and a Rab protein as a substrate has been proven to assess REP1 function.

Here we describe an *in vitro* potency assay using Rab6a to test AAV vectors for choroideremia gene therapy. **Methods** Cultured HEK293 cells were treated with AAV2/2-REP1 at a range of multiplicities of infection (MOI) or left untreated as a control. Total cell lysates were prepared in prenylation buffer at day 5 post-transduction. Prenylation reactions were set up using a biotinylated lipid donor, and Rab27a or Rab6a as substrates. Reaction products were run on a SDS-PAGE gel and analysed by immunoblot against human REP1 and actin as internal loading control. The biotinylated substrate was detected using streptavidin-HRP. **Results** The treatment with AAV2/2-REP1 showed that REP1 protein is overexpressed in a dose-dependent manner by immunoblot analysis. Additionally AAV2/2-REP1-treated cell lysates showed a greater incorporation of lipid donor into both substrates tested, when compared to untransduced controls, indicating that the REP1 delivered by the transgene is directly and proportionally functional. The signal obtained for lipidated product using Rab6a was higher than using Rab27a, which makes Rab6a a more sensitive substrate to detect prenylation levels *in vitro*. **Significance** Our data demonstrates that an *in vitro* prenylation assay using biotinylated lipid donors and Rab6a as a substrate is an effective method to assess the effectiveness of AAV2/2-REP1 for gene transfer and therefore can be used as a potency assay for testing clinical grade vectors for choroideremia gene therapy.

Synthetic/Molecular Conjugates and Physical Methods of Gene Delivery II, Oligonucleotide Therapeutics II

303. Metabolic Stability and Expression of Novel Double Stranded mRNA Polyplexes for Liver Transfection In Vivo

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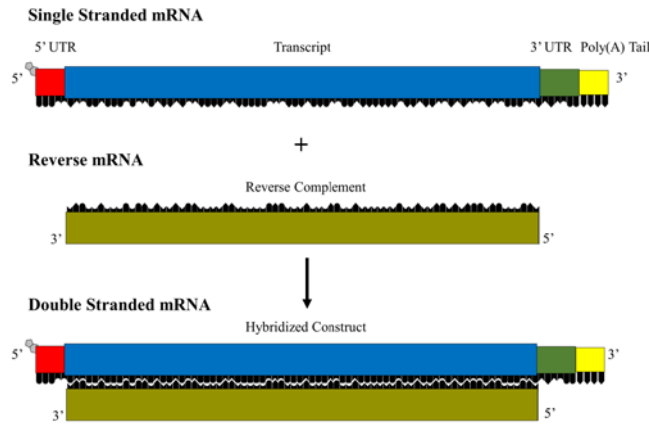
In vivo transfection of liver hepatocytes using mRNA potentially offers an advantage over delivering plasmid DNA because it eliminates the need for mRNA delivery to the nucleus. However, the metabolic instability of mRNA in the circulation limits the potency of mRNA nanoparticle gene delivery systems. The present study reports that hybridization of mRNA with a complementary reverse strand mRNA results in a novel double stranded (ds) mRNA possessing dramatically increased serum stability. Hydrodynamic dosing of ds mRNA nanoparticles demonstrates equivalent translational efficiency to single stranded (ss) mRNA nanoparticles. Optimal ds mRNA mediated gene expression in liver was achieved by tailoring the length of the reverse mRNA strand. The circulatory stability of ds mRNA nanoparticles was also found to be significantly greater than ss mRNA nanoparticles as determined by gene expression in the liver. In vitro transcription of reverse mRNA allows incorporation of unnatural nucleotide substrates, affording the opportunity to further stabilize and functionalize ds mRNA to improve delivery. The development of this novel form of metabolically stabilized and translationally active ds mRNA provides a new platform to advance applications of mRNA nanoparticle delivery to achieve gene expression and genome editing.



304. Topical and Systemic Administration of Chemically Modified *HCFTR* mRNA Restores Lung Function in a Mouse Model of Cystic Fibrosis

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Objective: Cystic fibrosis (CF) is the most common life-limiting genetic disease among Caucasians, and is caused by mutations in the underlying disease-conferring gene *CFTR*. Due to the genetic nature of the disease, gene therapy is a vital option for treatment. However, in the setting of CF, gene therapy resulted in low efficacy in various clinical trials, and thus demands a viable alternative. Transcript replacement therapy using chemically modified mRNA is such an option as the delivery of h*CFTR* mRNA restored the function of CFTR channel *in vitro*. In the current study we evaluated the therapeutic benefit of chemically modified h*CFTR* mRNA *in vivo*. **Methods**: hCFTR mRNA was produced with two well described chemical modifications along with unmodified variant. Gut corrected $CFTR^{-/-}$ mice received chemically modified h*CFTR* mRNA encapsulated in chitosan-coated PLGA nanoparticles by intra-tracheal spraying (n=5), and intravenous injection (n=5) targeting the lungs. The untreated control received mock mRNA (n=5). CFTR expression in lungs was measured by hCFTR ELISA. Channel restoration was screened by chloride assay by checking chloride concentration in saliva and Lung function was evaluated using FlexiVent. Immunogenicity of chemically modified h*CFTR* mRNA was investigated *ex vivo* in whole blood and also *in vivo* (n=3) after 6h, 24h and 72h.**Results:** In CFBE (cystic fibrosis bronchial epithelial) cells, expression of functional h*CFTR* mRNA was observed by flow cytometry and western blot. Channel function *in vitro* was demonstrated by halide sensitive YFP assay. Chemically modified h*CFTR* mRNA was observed to reduce chloride concentration in saliva compare to $CFTR^{-/-}$ mice or mock-treated $CFTR^{-/-}$ mice. Intriguingly, untreated $CFTR^{-/-}$ showed increased airway resistance and decreased compliance compared to wild-type $CFTR^{+/+}$ mice - normal lung function was restored after either i.t. or i.v. treatment with chemically

modified h*CFTR* mRNA. **Conclusion**: Our results support the notion that transcript therapy of chemically modified h*CFTR* mRNA is a potential alternative therapy for the treatment of CF.

305. XT-101 PLGA/Human IL-10v Plasmid Promotes Improved Quality of Life in Companion Dogs with Osteoarthritis

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Osteoarthritis (OA) is a disease of unknown etiology that manifests as significant impairment in joint function resulting from degeneration and destruction of tissues within the synovial capsule. Over 25 million sufferers in the US, and millions more world-wide, are poorly treated by current therapeutic options that target symptomatic relief. Importantly, this disease is also prevalent in the veterinary population and is especially common in dogs, cats, and horses. An important component of OA in all species is the production of proinflammatory cytokines within the joint capsule and the resultant alteration of cell functions within the joint. Interleukin-10 (IL-10) can reverse proinflammatory cytokine effects, but must be provided long-term for efficacy. IL-10-encoding plasmid-based gene therapies can provide long-term efficacy in models of neuropathic pain, another chronic condition driven by derangements in proinflammatory cytokine function. Here we describe the results of a pilot open-label study examining a PLGA-encapsulated formulation of this plasmid-based therapy, XT-101, using companion dogs with OA as a disease model. We demonstrate that XT-101, encoding a proprietary variant of human IL-10, delivered intra-articularly to dogs is well-tolerated and can provide long-term improvements in overall quality of life and joint function in these animals. Because of the similar natural history and progression of OA in human and veterinary populations, these data are supportive of translation of IL-10-based therapies into the clinic in both populations.

306. Novel Poly(Sarcosine) Click Shielding Agents Improve Circulation of Redox-Sensitive siRNA Lipo-Polyplexes *In Vivo*

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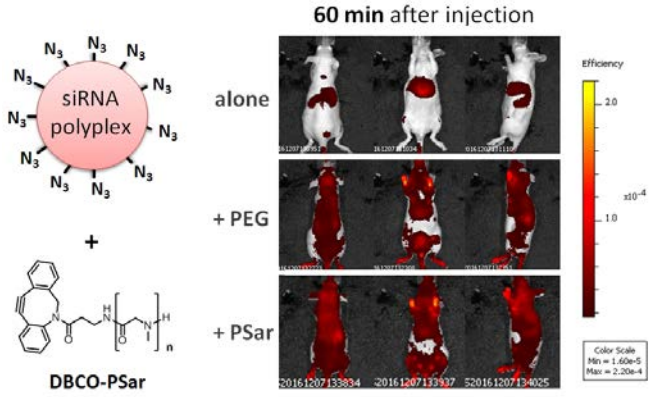
Lipo-oligomers have been proven to be very potent siRNA carriers based on stable electrostatic and hydrophobic complex formation and endosomal membrane destabilization. Although high stability of siRNA polyplexes is desirable in the extracellular space and cellular uptake, intracellular disassembly is important for the cytosolic release of siRNA. To improve the release, bioreducible sequence-defined lipo-oligomers were synthesized by solid-phase assisted synthesis using the Fmoc-succinoyl-cystamine disulfide building block for precise positioning of a disulfide unit between a lipophilic diacyl domain and an ionizable oligocationic nucleic acid binding unit. Reducible siRNA lipo-polyplexes show higher gene silencing efficacy and lower cytotoxicity than their stable analogs, consistent with glutathione-triggered siRNA release and reduced lytic activity (Klein et al, *Nanoscale* 2016, 8, 18098-18104). In the current work, optimized redox-sensitive lipo-oligomers were extended by azido functionality to serve as reactants for click chemistry. After formation of siRNA lipo-polyplexes, the azido groups were used to covalently modify the nanoparticle surface with DBCO-modified poly(sarcosine). Polymerized sarcosine (N-methylglycine) is hydrophilic and biodegradable. These PSar-modified lipo-polyplexes show excellent shielding properties in terms of reduction of zeta potential and cellular uptake. *In vivo* bioimaging studies were performed in mice to assess the distribution of intravenously injected siRNA lipo-polyplexes with 8 kDa poly(sarcosine) or 5 kDa poly(ethylene glycol) shields. Both formulations showed a much-expanded *in vivo* distribution as compared to unshielded lipo-polyplexes.



307. Mono-Uridylated siRNA Provides Powerful Therapeutic Tool for Colon Cancer

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The non-templated nucleotides to the 3' ends of small RNA molecules can have a profound impact on their stability and biological function. Evidence accumulated over the past few decades has established roles for uridylation and adenylation in small RNA stabilization and degradation. 3'-end formation of small RNAs occurs through a delicate balance between the removal and the addition of nucleotides. By sequencing transfected small RNAs of total RNA and associated with DICER-containing RNP, we observed that the majority of non-template mono-uridine in 3' end of small RNAs were associated with DICER. It is currently believed that Dicer may be a haploinsufficient tumor suppressor, and deletion of Dicer has been evidenced in various human cancers. In this context, the hemizygous loss of Dicer has been observed in various tumor types, and low levels of dicer expression are known to predict poor survival of patients with chronic lymphocytic leukemia, breast, lung, ovarian, nasopharyngeal, colorectal and bladder cancer. Furthermore, low levels of Dicer expression occur in colorectal cancer liver metastasis. We found mono-uridylated siRNA associated with Dicer can inhibit the target gene for long time. The naked siRNA disappeared a gene-knockdown efficiency on 3 days after transfection but mono-uridylated siRNA had significant activity

for 8 days after transfection. In our study, the mechanism of action for mono-uridylated siRNA was involved with EXOD protein stimulated the long-term stability of siRNAs in colon cancer cells. These finding demonstrate the therapeutic potential of mono-uridylated siRNAs for the treatment of colon cancer.

308. Chemical Functionalization of Double Stranded mRNAs

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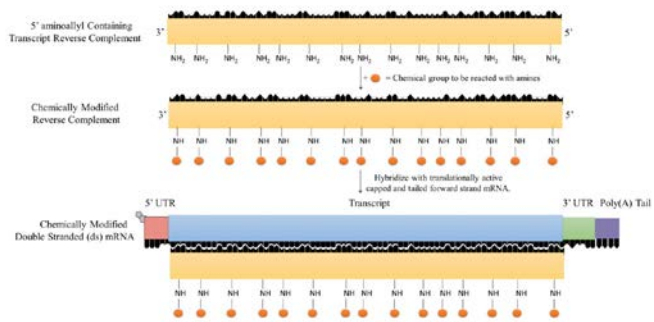
We have recently developed double stranded (ds) mRNA by hybridizing (ss) mRNA with a complimentary reverse strand to increase metabolic stability. In the present study, we demonstrate that the reverse strand can be used as a carrier to accept chemical modification to impart desired properties necessary for efficient delivery. Efficient incorporation of 5'-aminoallyl uridine and/or cytidine during in vitro transcription resulted in reverse mRNA with approximately 450 or 900 primary amines. Complete functionalization of primary amines with acetyl, maleic acid, thiol-acetate and PEG was demonstrated by agarose gel electrophoresis band shift assay. Functionalized reverse RNAs retained the ability to hybridize with translationally active forward mRNA to form ds mRNA. The metabolic stability of chemically modified ds mRNA was analyzed by gel electrophoresis following RNAse digestion. Hydrodynamic dosing of chemically modified ds mRNAs into mice followed by bioluminescence imaging of luciferase expression in the liver was used to assess the influence of reverse strand chemical modification on gene expression. The results establish that positively and negatively charged functionalized reverse RNA resulted in ds mRNA that was less translationally active compared to neutrally charged substitution. However, positively charged ds mRNA was significantly more stable to digestion with RNAse compared to neutrally charged ds mRNA. Substitution and chemical modification of uridine in reverse RNA results in more translationally active ds mRNA compared to modification of cytidine. Double substitution of cytidine and uridine in reverse RNA strands followed by chemical modification with acetyl results in translationally active ds mRNA. Non-reversible PEGylation blocks ds mRNA translation whereas reversible PEGylation was relatively more translationally active. The ability to partially substitute 5'-aminoallyl uridine and cytidine into reverse RNA by blending substrates during in vitro transcription was used to control the number of chemical substitution sites to influence metabolic stability and translation efficiency. This first-ever demonstration of a novel chemical modification of reverse mRNA used to hybridized with translationally active unmodified forward mRNA provides a chemical tool-box to develop more chemically diverse ds mRNA.



AAV Vectors II

309. Mapping of Conformational Epitopes of Monoclonal and Polyclonal Antibodies Against AAV Capsids by an Immunoprecipitation-Seq (IP-Seq) Technology

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High prevalence of pre-existing neutralizing antibodies (NABs) against AAV capsids in humans poses a significant barrier to successful AAV vector-mediated gene therapy. There has been strong enthusiasm about developing stealth AAV vectors that can evade NABs. However, creation of such AAVs requires more comprehensive information about NAB epitopes, which currently remains very limited as there has been no method of mapping epitopes for polyclonal anti-AAV capsid antibodies present in animal and human sera in an easy and effective manner. Here we devise an IP-Seq technology that can effectively map conformational epitopes of monoclonal and polyclonal antibodies against AAV2 capsids in multiple samples at the same time by multiplexed Illumina barcode sequencing. In this method, we produced a DNA-barcoded AAV9-hexapeptide (HP) scanning capsid mutant library comprising a total of 153 AAV9-HP mutants in addition to the wild-type AAV9 (a negative control), as well as the wild-type AAV2 and the AAV2R585E heparin binding-deficient mutant (positive controls). Each AAV9-HP mutant contained a substitution of 6 consecutive amino acids derived from different regions of the wild-type AAV2 capsid so that various HP regions in the AAV2 capsid can be displayed on the heterologous AAV9 capsid in a nearly native quaternary structure. The HP scanning of the AAV2 capsid was performed at a two amino acid interval creating 153 overlapping HPs. These AAV9-HP mutants cover the majority of the AAV2 capsid amino acids that differ from those of the AAV2 capsid. The IP-Seq procedure is composed of the following three steps: (1) IP of the AAV9-HP library (AAV viral particles containing DNA-barcoded genomes) with monoclonal or polyclonal antibodies present in commercially available reagents or animal sera; (2) extraction of DNA-barcoded genomes from immunoprecipitates; and (3) Illumina barcode sequencing of the recovered viral genomes followed by a bioinformatic analysis. The IP procedure required optimization experiments, which revealed the combination of A/G protein-coated magnetic beads and blocking with 2% BSA to be the best condition for lowering non-specific binding without restricting binding of the library clones. Using the AAV9-HP mutant library and the IP-Seq, we could successfully identify amino acids that are contained in the known epitope of the A20 mouse monoclonal antibody against intact AAV2 particles, which demonstrates proof-of-principle of the method. Subsequently, using the same approach, we identified epitopes of polyclonal anti-AAV2 capsid antibodies in the sera of AAV2-immunized mice. The identified epitopes include 261-SSQSGA-266 (the same as the epitope of A20) and 451-PSGTTT-456, which are shared with multiple serum samples. We are currently extending our technique to human polyclonal antibody epitope mapping. However, our initial ELISA screening of human sera has shown that many anti-AAV2 antibody-positive human serum samples are also positive for anti-AAV9 antibodies. This makes it not possible to apply our technique directly to human samples because

effective mapping of anti-AAV2 antibody epitopes is possible only when samples do not bind AAV9. To cope with this problem, we have successfully developed, and confirmed by ELISA, an anti-AAV9 antibody neutralizing technique by incubating human sera with an excess amount of AAV9 particles before subjecting the sera to IP-Seq. This promising result leads us to a future plan to screen hundreds of human sera to find suitable sera samples for IP-Seq and identification of polyclonal human antibody epitopes. Thus, the IP-Seq we have devised is an effective approach to mapping conformational anti-AAV capsid antibody epitopes and future development of anti-AAV neutralizing antibody-escaping mutants.

310. Hypervariable Region IX Is a Major Domain Responsible for Adeno-Associated Virus 3B's Species Specificity

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Adeno-associated virus 3B (AAV3B) efficiently transduces both human hepatocyte cell line Huh7 and nonhuman primate liver; however, transduction of murine liver is very poor. To determine which regions of the AAV3B capsid are responsible for species-specific transduction, we engineered a series of vectors where the hyper-variable regions (HVRs) of AAV3B and AAV8 were exchanged. Swapping HVR VIII from AAV3B into AAV8 increased Huh7 transduction by ~3 fold, while other individually replaced HVRs had no effect on transduction. However, AAV8 carrying HVR VI and HVR VIII both derived from AAV3B increased Huh7 transduction ~10 fold over that of wild type AAV8. Likewise, AAV8 with AAV3B-derived HVR IV and HVR VIII exhibited improvement of transduction but to a lesser degree. Other HVRs tested in combination with AAV3B-derived HVR VIII had no positive effect on transduction. Intriguingly, HVR VI is not exposed on the AAV8 capsid surface. The importance of HVR VIII on AAV3B's Huh7 transduction efficiency was further confirmed using an AAV3B vector with an AAV8-derived HVR VIII, which demonstrated a dramatic decrease (~50 fold) in transduction of Huh7 cells. The HVR VIII of AAV3B differs from AAV8 by seven amino acids that cluster into four patches. By exchanging each of these four patches from the AAV3B capsid into AAV8, we showed that surface-exposed positive charges in this region are likely involved in AAV3B's high Huh7 transduction efficiency. AAV3B transduces murine cell lines less efficiently than AAV8. By comparing the transduction of a human cell line with murine cell lines for AAV3B HVR mutants (where HVR I-IX of AAV8 were individually exchanged into AAV3B), we found that the HVR IX region is key to controlling AAV3B's species-specific transduction, which we confirmed *in vivo*. Transduction efficiency of the mutant AAV3B.8VR9 (AAV3B with its HVR IX replaced with that of AAV8) vector was assessed in C57BL/6 male mice. Briefly, mice were injected systemically (via the tail vein) with 10^{11} genome copies of AAV3B.8VR9 mutant vector expressing human factor IX (hF9) under the TBG promoter. One week after vector injection, hF9 expression was >20 fold higher than that conferred by AAV3B vector. These *in vivo* data corroborates our previous AAV2 study (Tenney *et al.*, 2014, PMID: 24725949), which showed that replacing the AAV2 HVR IX (which is the same as AAV3B) with HVR IX from AAV8 significantly increased transduction of murine liver. Our results suggest that careful assessment of vector performance is warranted when evaluating AAVs carrying the AAV3B-HVR IX sequence in mice.

311. AAV9-Utrophin Prevents Myonecrosis in Dystrophic Mice and Dogs

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A majority of the sporadic mutations causing Duchenne muscular dystrophy (DMD) result in a deletional frameshift and absence of the giant cytoskeletal protein dystrophin. To optimize the function of a non-immunogenic substitute for dystrophin small enough for systemic delivery, we used a phylogenomic approach to reconstruct the evolutionary history of dystrophin's orthologs and closest paralog, utrophin. Sustained expressed transduction of striated muscle with an AAV9 vector (1.0×10^{14} vg/kg) encoding a reverse-engineered micro(μ)Utrophin conferred histologically complete protection against myonecrosis and normalization of the level of serum CK (4744 ± 2431 vs. 634 ± 413 U/L) in treated dystrophin-deficient mdx mice as well as significantly improved muscle function both *in vivo* and *ex vivo*. In dystrophic dogs, AAV9μUtrophin (2.0×10^{14} vg/kg) restored normal sarcoglycan/dystroglycan expression/localization as well as reduced the level of serum CK without provoking a detectable immune response to transgene product. The exceptional functionality and immunological stealth of the optimized AAV9μUtrophin offers the unique combination of efficacy and safety needed for clinical translation.

312. Targeting Visceral Fat by Intraperitoneal Delivery of Novel Recombinant Adeno-Associated Viral Vectors Restricting Off-Target Transduction in Liver

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Recombinant adeno-associated viruses (rAAVs) has emerged as attractive and safe vehicles for gene therapy. Naturally occurring rAAV serotypes are ineffective for gene delivery to the adipose tissues. We recently report that an engineered hybrid rAAV serotype Rec2 achieves high transduction efficacy in adipose tissues via direct injection to subcutaneous fat. Here we demonstrated that intraperitoneal injection of Rec2 vector led to widespread and high level transgene expression (EGFP) in visceral fat depots including epididymal, mesenteric and retroperitoneal fat pads at a dose at least 5-10 fold lower than the commonly used doses for systemic gene delivery. To achieve adipose-preferable transduction, we developed a rAAV expression plasmid

harboring two expression cassettes, one using chicken beta-globin promoter to drive transgene expression, and the other using albumin promoter (a liver specific promoter) to drive a microRNA targeting the WPRE sequence that only exists in this rAAV vector. This dual cassette vector resulted in highly selective transduction of visceral fat while severely restricting off-target transduction of liver. In a proof-of-concept study, intraperitoneal administration of the liver-restricting Rec2 vector harboring therapeutic gene leptin ($4x10^{10}$ vg/mouse) robustly alleviated the obesity and metabolic syndrome of *ob*/*ob* mice over 9-week period. This study provides a highly effective gene delivery vehicle with improved specificity and safety to genetically manipulate fat for basic research as well as to treat genetic and acquired diseases such as obesity, metabolic syndromes, and cancer. This work was supported by NIH grants CA-166590, CA-178227, CA-163640 to L.C.

313. Large-scale Molecular Epidemiological Analysis of AAV in the Chinese Cancer Patient Population

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In 1965, adeno-associated virus (AAV) was discovered as a contaminant in adenovirus preparations. Now after several decades, AAV has become the most promising viral vector platform for delivering therapeutic gene products in vivo. Numerous studies on recombinant AAV (rAAV) have demonstrated its multi-functionality and technological impact on preclinical and clinical research. Yet, these same studies also highlight rAAV's potential shortcomings. Particularly, a recent controversial report suggesting that wild-type AAV is directly linked to hepatocellular carcinoma has cautioned the safety of rAAV-based therapies. To address whether wtAAV is a potential tumor risk factor, we have performed molecular epidemiological analysis of AAV in the Chinese cancer patient population receiving care at West China Hospital (Chengdu, China), by far the largest population studied to date (N=413). Resected tumor samples reflecting nine different carcinoma types (breast carcinoma, rectal cancer, pancreas carcinoma, brain tumor, hepatoid adenocarcinoma (HAC), hepatocellular carcinoma (HCC), gastric carcinoma, lung squamous and adenocarcinoma) were collected from patients diagnosed by radiological and biopsy examination. Both neoplastic masses and adjacent non-lesion tissues were obtained to directly investigate AAV infection and tumorigenesis. By using "signature" PCR detection of AAV capsid proviral sequence (250 bp), we discovered that over 80% of tissues were AAV positive, present in all nine types of carcinoma examined. Interestingly, different carcinoma/tissue types varied in AAV positivity. For example, lung squamous carcinomas were 98% positive for AAV (96% in adjacent non-lesion tissues), while lung adenocarcinomas were 58% positive (56% in adjacent non-lesion tissues). Importantly, we found no significant difference in AAV detection in patient matched tumor and adjacent non-lesion tissues in incidence and abundance. In total, we detected proviral sequences for serotypes AAV2, AAV2/3-hybrid, AAV6, and AAV8 among all tissues. The most frequently detected serotype was AAV2/3, which was found in all tissue samples. Notably, lung squamous carcinomas and adjacent non-lesion tissues are 100% positive for AAV2/3. Conversely, AAV6 was only detected in several gastric carcinoma samples. No specific AAV sequences were overrepresented in tumor samples as compared with normal tissues.

In summary, this body of ongoing work critically adds to the epidemiological profile of AAV in humans, and provides important and informative findings that may be helpful for rAAV-based clinical studies, gene therapeutics, and its overall safety in human applications.

W.Q., G.X., P.W.L.T. and C.W. are Co-first authors; Y.W., T.R.F. and G.G. are Co-corresponding authors.

314. Accurate Identification and Quantification of DNA Species by Next Generation Sequencing in Adeno-Associated Viral Vectors Produced in Insect Cells

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Recombinant adeno-associated viral vectors (rAAV) have proven excellent tools for the treatment of many genetic diseases and other complex diseases. However, the illegitimate encapsidation of DNA contaminants within viral particles constitutes a major safety concern for rAAV-based therapies. Moreover, the development of rAAV vectors for early-phase clinical trials has revealed the limited accuracy of the analytical tools used to characterize these new and complex drugs. While most published data concerning residual DNA in rAAV preparations have been generated by quantitative PCR, we recently developed a novel single-strand virus sequencing (SSV-Seq) method for quantification of DNA contaminants in AAV vectors produced in mammalian cells by next-generation sequencing (NGS). Here, we describe the adaptation of SSV-Seq for the accurate identification and quantification of DNA species in rAAV stocks produced in insect cells. We found that baculoviral DNA was the most abundant contaminant, representing less than 2.1% of NGS reads regardless of serotype (2, 8, or rh10). *Sf*9 producer cell DNA was detected at very low frequency (≤0.03%) in rAAV lots. Advanced computational analyses revealed that (i) baculoviral sequences close to the ITRs preferentially underwent illegitimate encapsidation, and (ii) single nucleotide variants (SNVs) were absent from the rAAV genome. The high-throughput sequencing protocol described here enables effective DNA quality control of rAAV vectors produced in insect cells, and is adapted to conform with regulatory agency safety requirements

315. Assessment of the Immunogenicity Against AAV with the LK03 Chimeric Capsid Variant in the Human Population

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Background: AAV vectors currently represent the leading technology for clinical translation of liver-directed gene therapy. AAV8 vectors have pioneered clinical successes in hemophilia B with a mild increase of plasma factor IX from less than 1% to up to 6%. However, other liver monogenic disorders might require a higher rate of hepatocyte transduction to achieve a significant clinical benefit. To improve hepatocytes transduction, alternative re-engineered capsids have been developed by DNA shuffling, such as AAV-LK03 composed of 5 different parental wild type capsids. Indeed, AAV-LK03 targets human liver cells with an efficiency one log higher than AAV8, and has also improved transduction compared to AAV3B. Preexisting humoral immunity against the AAV capsid has the ability to significantly reduce gene delivery and long-term expression. Therefore, to explore the possibility of clinical translation, we have investigated the prevalence of neutralizing factors against AAV-LK03, by screening sera from a large cohort of pediatric and adult donors. Methods: Serum samples from 4 different age cohorts (up to 6 months old, 7m-2yo, 3-17yo, and 18-50yo) were assessed. HuH7 cells were transduced with single-stranded AAV-LK03-hAAT-GFP (MOI of 2000) mixed with serial two-fold dilutions (1:5 to 1:1280) of the heat-inactivated serum samples and incubated for 1 h at 37°C. The neutralising antibody titer was defined as the dilution, which inhibits the transduction of 50% or more hepatocytes. The sera were judged positive for a neutralising titer of 1:5. Results and Discussion: The seroprevalence of neutralizing antibodies against AAV-LK03 was 40 % in the age group up to 6 months old (n=10), 32 % in the age group 7m-2yo (n=38), 26 % in the age group 3-17 yo (n=87), and 44 % in the age group 18-50 yo (n=55). Considering that the low seroprevalence of pre-existing anti-AAV-LK03 neutralising antibodies is similar to AAV8, AAV-LK03 might be a promising liver-tropic vector for clinical translation. The ability to more easily evade human immunoglobulins is also an advantage, which might be of interest in clinical use allowing a higher percentage of patients to benefit from it.

316. Enhanced CNS Specificity of Adeno-Associated Viral Vectors Created by DNA Shuffling and Directed Evolution

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Recombinant adeno-associated virus (AAV) is a commonly-used gene therapy vector for central nervous system (CNS) disorders due to its impressive safety profile, nonpathogenic nature, and efficiency in transducing a wide range of cell types (particularly neurons). AAV9 administered systemically can cross the blood-brain barrier and confer widespread gene transfer to the CNS, with axonal transport occurring in both anterograde and retrograde directions. Intra-CSF delivery of AAV is also a relevant translational approach, given recent human trials utilizing this route for Giant Axonal Neuropathy and Neuronal Ceroid Lipofuscinosis type 6. Intra-CSF administration (via cisterna magna (ICM) or lumbar cistern) offers an alternative to the intravenous route, resulting in relatively lower peripheral organ biodistribution, circumvention of moderate levels of blood neutralizing antibodies, and potentially lower efficacious doses. While clinically-relevant CNS gene transfer can be achieved using intrathecal (IT) AAV9 administration, this vector and approach does have several potential limitations, depending on specific treatment needs: considerable biodistribution to peripheral organs; AAV9 transduces a mixture of neurons and glia throughout the CNS; and the vector does not distribute homogeneously throughout the CNS. Our lab has utilized AAV capsid DNA shuffling and directed evolution to create a library of novel AAV capsids with potentially unique features. This library was derived from a variety of AAV serotypes and incorporates additional random mutagenesis. Following lumbar IT injections in mice, novel capsids were selected based on their ability to reach deeper brain structures. The input library was biased towards AAV8 sequence in the C-terminal half of the capsids and selected capsids showed little diversity in this region, but were highly diverse in the N-terminal half. A total of 64 novel capsid sequences (referred to as the AIM collection) were recovered across 3 rounds of selection. To evaluate the biodistribution and cellular tropism of the recovered capsids, each was packaged with a self-complementary CBh-GFP genome and delivered via intra-CSF administration (ICM or IT) in adult mice. Mice were harvested 3-4 weeks post-injection for qPCR biodistribution of vector DNA to peripheral organs and immunofluorescence (IF) staining of GFP expression within the CNS. Relative to AAV9, IF staining showed a range of neuronal (versus glial) tropism within the CNS, along with transduction efficiency of several clones that is equivalent to AAV9. One clone (ITcordNr3.03) was particularly interesting in that it had an overall CNS transduction efficiency that is 2-3 fold higher than that of AAV9. Further, the peripheral biodistribution of this clone was highly reduced (~1000-fold for liver) relative to AAV9. These findings suggest that some of the recovered capsid clones might be useful alternatives to AAV9 in applications requiring enhanced CNS specificity, altered neuronal/glia tropism, and/or reduced peripheral organ biodistribution. Further, since these capsids are composed of a mixture of AAVs, there is potential for them to be used in patients seropositive for AAV9.

317. Direct Head-to-Head Evaluation of RAAV Vectors Manufactured in Human *vs* Insect Cells

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The major drawback of the Baculovirus system for rAAV manufacturing is a relatively poor infectivity of many serotypes produced in Sf9 cells. In fact, most of the Bac-derived serotypes, with few exceptions, characterized by altered capsid composition and lower potency. Hereby, we describe a significantly upgraded OneBac system incorporating a modified *cap* helper gene encoding adjustable ratios of VP1:VP2:VP3 capsid proteins. The ratio could be fine-tuned by utilizing a serotype-specific attenuated Kozak sequence and a leaky ribosome scanning. By way of example, rAAV5 and rAAV9 were produced and comprehensively characterized, side-by-side with HEK 293 derived vectors. The re-designed rAAVs are characterized by significantly higher biological potencies, even in a comparison to HEK 293-manufactured rAAVs mediating, in case of rAAV5, 4-fold higher transduction of brain tissues in mice. Furthermore, we conducted an extensive analysis of encapsidated single-stranded viral DNA using Next-Generation Sequencing (NGS) demonstrating significantly lower levels of collaterally packaged contaminating DNA for rAAV5 produced in Sf9 cells. Thus, the latest version of OneBac system yields rAAV vectors of superior infectivity and exceptional purity providing a scalable platform for GMP grade vector production.

318. Transduction of Rhesus Macaque Lung by AAV1 of Rhesus Macaque Lung by AAV1

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The major hurdle with gene therapy is expression of enough CFTR protein to be therapeutic. In order to address this, we utilized a dual reporter assay based upon firefly (FL) and renilla (RL) luciferase cloned into AAV1 & 5 vectors. Two male and two female healthy Rhesus monkeys were exposed by trans-oral Penn Century microsprayer delivery to an aerosol containing both AAV1-CB-FL and AAV5-CB-RL. CB refers to the chicken β-actin promoter. The studies were sponsored by NHLBI Gene Therapy Resource Program (GTRP) and conducted at the Lovelace Respiratory Research Institute. The vector combination was formulated on the day of dosing by combining, as provided by the U. of Mass, Vector Core, 13.5 ml of the AAV5-CB-RL (1.2×10^{13} gc/ml) with 16.2 ml of the AAV1-CB-FL (1.0×10^{13} gc/ml), to provide a mixture with 0.54 gc/ml of each vector. Animals were observed for clinical signs of toxicity, and body weight. The animals were euthanized at 45 days post-exposure. Seventeen lung samples were collected for analysis of luciferase reporter gene expression (RL versus FL) at the U of Iowa and for vector expression by PCR (U of Florida). Importantly, the only significant clinical observation was a foot pad laceration, which was not a test article-related finding. All animals gained weight, as expected, during the study. These results show again that AAV1 vector delivery is safe. The vector genomes measured in each lung region at necropsy showed that the vector was widely distributed throughout the lung by the microsprayer demonstrating conclusively that our droplet size is sufficient to support widespread distribution. Average PCR data for 17 lung samples from four monkeys show clearly that there is a dramatic difference between the ability of AAV1 and 5 to infect the lung with AAV1 being approximately 10-fold more effective than AAV5. Despite the small number of monkeys, the data also clearly show better luciferase transduction with AAV 1 than with AAV5. The levels of neutralizing antibody (measured at U. Penn) increased dramatically in all animals between the pre-study time point (monkeys chosen for the study had undetectable titers) and the time of necropsy. Considerably higher titers were observed for AAV5 than for AAV1. These results provide justification for our choice of AAV1 for lung delivery given that it had a greater infection efficiency and transduction and lesser propensity to induce neutralizing antibodies than did AAV5. Funded by CFF and NHLBI.

319. Ultracentrifugation-Free Chromatography-Mediated Purification of Recombinant Adeno-Associated Virus Serotype 9 (rAAV9)

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[Background] The current production of rAAV from the transfected cell lysate and purification based on CsCl or iodixanol density ultracentrifugation is not scalable. Although rAAV9 is promising therapeutic vector for genetic neuromuscular disorders, the scalable purification method for this vector has not yet been established. We previously reported an ultracentrifugation-free purification method for rAAV1, although purification conditions of the rAAV varies according to serotypes. Therefore, in this study, we elaborate the novel chromatography-mediated method for purification of rAAV9 from the serum-free culture supernatant with ultracentrifugation-free technique.

[Methods] rAAV9 was produced by the triple-transfection to HEK293EB cells (stably expressing the *E1* gene region of adenovirus type 5 (*E1A*, *E1B19K*, and *E2A*) and *Bcl-x_L* gene) in serum-free medium with polyethyleneimine (PEI). Five days later, the culture

supernatant was concentrated by the hollow fiber tangential flow-filtration (TFF) system. After reducing protein debris by 1/3-saturated ammonium sulfate (1/3 AS) precipitation, rAAV9 was precipitated in 1/2 AS solution. After the precipitated rAAV9 was dissolved in 3.3 mM MES, 3.3 mM HEPES, 3.3 mM sodium acetate (MHN) buffer (pH8.0) containing 50 mM NaCl and 0.01% Pluronic F-68, it was loaded to quaternary amine charged anion-exchange column. The passed through fraction containing rAAV9 was finally purified by gel-filtration chromatography. We examined for 3 trials with *egfp* transgene (scAAV9-CB-EGFP), and for each 1 trial with other transgenes (scAAV9-CB-red-firefly-luciferase (scAAV9-CB-RFLuc) or ssAAV-CMV-RFLuc) with this protocol. The physiological and biological properties of the purified rAAV9 were characterized by qPCR, negative stain by electron micrograph, flow cytometry by EGFP, western blot and SDS-PAGE.

[Results] The purified scAAV9-CB-EGFP displayed three major protein bands (VP1, VP2, and VP3) on SDS-PAGE and 96% on average of rAAV9 particles were contained fully packaged viral genomes according to electron micrographic analysis (n=3). The resultant genomic titer of the purified rAAV9 was 2.5 x 10^{15} v.g. on average from as few as 3.2 x 10^{9} HEK293EB cells (n=3). Of the purified rAAV9 particles with other transgenes, 92.7% (4341 / 4684 particles) at scAAV9-CB-RFLuc and 96.2% (5350 / 5561 particles) at ssAAV9-CMV-RFLuc also contained fully packaged viral genomes as determined by negative-stain electron micrographic analysis. The resultant titer of scAAV9-CB-RFLuc was 3.9×10^{15} v.g. and of ssAAV9-CMV-RFLuc was 1.8×10^{14} v.g.

[Conclusion] Ultracentrifugation-free chromatography-mediated purification of rAAV9 from the culture supernatant is a major breakthrough. We obtained rAAV9 by this protocol with high titer, high purity and minimum contamination of empty particles. We determined a buffer condition to separate empty particles and rAAV9 by anion-exchange column chromatography based on isoelectric point, which leads to establish an ultracentrifugation-free technique. This novel chromatography-based method facilitates scaled-up current good manufacturing practice (cGMP)-compliant production of rAAV9 for clinical applications in gene therapy in the future.

320. P5 Sequences Is an Enhancer for AAV Vector Packaging

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One of the major obstacles for broad use of recombinant AAV vectors has been the time-consuming, labor intensive production process. The common method for producing AAV vectors requires co-transfection of plasmids carrying all the necessary cis and trans components into HEK 293 cells. Since it is a widely-accepted principle that the AAV ITRs are the only cis-acting element that are essential in genome rescue, replication and packaging, great attentions have been paid to optimize the trans components for enhancing AAV production. Nevertheless, non-ITR cis-elements have been suggested to be involved in AAV replication and packaging. Here, we systemically investigate the role of P5 sequences in AAV vector production and its effect on transgene expression. Interestingly, vectors including a P5 element showed 3~4-fold higher packaging efficiency. The orientation and position of the P5 sequences in the AAV genome also affects the final vector yield. In addition, the nucleotide composition in the proximity of ITRs is another factor affecting the packaging efficiency. The effects of P5 sequences can vary depending vector DNA compositions as well. Presence of P5 element in the rAAV genome allowed more efficient expression of capsid proteins and replication of vector genomes. Furthermore, we screened to the key P5 sequences that shows the enhancing effect on AAV packaging. Besides the main determinant sequence (nucleotide 250-304 of AAV2), nucleotide 317-431 and 411-539 can increase AAV packaging as well. Finally, We demonstrated the P5 sequence in the rAAV genome had no negative effects on transgene expression both in vitro and in vivo. Our studies suggested the P5 element could be used as an enhancer to increase AAV yield.

321. HeLa Screening Platform Advancements to Accelerate Stable Selection of High Producing Recombinant Adeno-Associated Virus (rAAV) Cell Lines

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With increasing phase I/II clinical evidence that recombinant adeno-associated virus (rAAV) vectors hold promise for gene therapy, it is crucial to develop viable manufacturing processes for large-scale rAAV production. Stable suspension cell line generation for commercial-scale rAAV production is a preferred system due to scalability advantages relative to adherent models and higher batch-to-batch consistency relative to transient technologies. However, generating a quick, robust screening platform for high titer producer cell line development is challenging. An important step in efficient producer cell line generation is the early and accurate identification of high vector producers. Moreover, any improvement that minimizes the number of clones screened and reduces the development timeline is highly desired. We have previously described a three-tier clone selection process that has significantly reduced the development timeframe, but further advancements have been made. In an effort to improve the accuracy and ease of the early primary screen, we investigated the effects of media and well-plate format (adherent versus suspension) on clone selection for scale-up. Additionally, we have incorporated cell confluency measurements to approximate specific productivity and thus guide appropriate selection for scale-up to the secondary screen. Lastly, substituting shake flasks with a micro-bioreactor system in the tertiary screen has allowed for a higher throughput of cell lines with a significant reduction in user interaction and tightened cell system control to resemble large-scale bioreactor culture conditions. This evolution has also reduced scale-up time from secondary to tertiary screens due to the need for significantly fewer cells in the micro-bioreactor vessels. Overall, these advancements have led to the establishment of a HeLa screening platform with 1) an improved primary screen for better selection accuracy, 2) less user interaction to minimize error and work-related repetitive injuries, and 3) a tertiary screen that more closely represents the large-scale bioreactor manufacturing process for a final, rational clone selection.

322. Baculoviral hr Sequences Increase Baculovirus Stability and AAV Production Yield

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Adeno-associated virus (AAV) is a small, single-stranded DNA virus. It has been widely used as a gene delivery vehicle for studies in the fields of gene therapy, neuroscience, and cancer therapy. Traditionally, AAV was produced in mammalian cells through transient transfection method. Recent development in AAV production technology has demonstrated that AAV can be produced in insect cells with much higher productivity than in mammalian cells. However, recombinant baculovirus (rBV) carrying the AAV rep and cap genes exhibited instability upon repeated passages, which resulted in the loss of rep-cap sequences and therefore the AAV productivity. Since baculoviral homologous region (hr) sequences function as DNA replication origin, we reasoned that cloning these replication origins near the rep-cap genes could help replicate the rep-cap sequences and therefore stabilize the rBVs. Based on this reasoning, we cloned hr1 and hr2 respectively near the rep-cap expression cassettes at different locations and generated rBVs. After plaque purification and repeated passaging of these rBVs, we found out that these rBVs exhibited much better stability when compared with those rBVs without the cloned hr sequences. In addition, hr-containing rBVs can produce much higher yields of AAV vectors than those rBVs without the cloned hr sequences. The same improvements in rBV stability and AAV productivity were observed in all the AAV serotypes tested. However, when we cloned the hr sequence in the rBV containing the AAV genome near either 5'- or 3'-ITR , we did not observe any improvement in rBV stability or AAV productivity. Finally, we tested in vitro the infectivity of AAV vectors produced by rBVs with hr sequences and compared with that of AAV vectors produced by rBVs without hr sequences. Our results indicate that there is no difference between them in terms of infectivity. These results let us conclude that cloning the hr sequences near the rep-cap expression cassettes not only stabilize the rBVs and but also produce more AAV vectors without compromising the AAV infectivity.

323. Assessment of Biological Activity of DTX301 AAV Gene Therapy Product Using a Matrix Approach

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DTX301 is an adeno-associated virus (AAV) gene therapy product candidate under development for the treatment of ornithine transcarbamylase (OTC) deficiency. OTC deficiency, the most common urea cycle disorder, is caused by a deficiency in a liver enzyme responsible for detoxification of ammonia, which is formed as a metabolite of protein degradation. Individuals with OTC deficiency can build up excessive levels of ammonia in their blood, potentially resulting in neurological deficits and other toxicities. DTX301 entered a global, multi-center Phase 1/2 clinical trial to evaluate safety and efficacy in patients with OTC deficiency at the end of 2016 (NCT02991144).

AAV gene therapy products are complex biologics and their assessment involves a multi-step process. Thus, in the development of biological activity assays for DTX301, we have employed an incremental and multi-faceted approach to ensure adequate understanding of our product at the current stage of clinical development. A matrix approach was developed utilizing a set of *in vitro* assays, including $TCID_{50}$, sequencing of the transgene, and *in vitro* expression of OTC protein. OTC enzyme expression in Huh7 cells is determined using an in-cell Western (ICW) technique, with an antibody specific for human OTC. The ICW assay is performed as a relative potency assay, so full characterization of the reference standard is critical. These *in vitro* assays have been used to characterize an internal DTX301 reference standard which was qualified to demonstrate biologic activity in an *in vivo* model of OTC deficiency.

The mechanism of action of DTX301 is to restore OTC enzyme activity within the urea cycle via delivery of a gene therapy vector. This mechanism of *in vivo* action has been assessed in nonclinical studies with DTX301 in the *spf^{ash}* mouse model, a well-established model for OTC deficiency.

By characterizing the reference standard for the *in vitro* assays in a mouse model of disease, translational data on the potency relevant to the clinical endpoint can be obtained. The *in vivo* potency data and *in vitro* matrix assessment ensures the robustness of the characterization for the Phase 1/2 clinical study and establishes groundwork for assay development for Phase 3 and commercialization.

324. Enhancing the Efficacy of Acute Insulin Therapy to Improve AAV Transduction of Skeletal Muscle, Liver and Lung by Altering the *INSR* (Insulin Receptor) Levels

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Introduction: Our lab previously reported that an acute insulin therapy co-administered with AAV vector delivery to skeletal muscle and liver (mouse tissue and human cultured cells) increased transgene product 3-5 fold. This was not seen in lung. Further evidence showed that *INSR* (insulin receptor) gene expression levels were dramatically increased (7-25 fold) in liver and skeletal muscle tissues/cells compared to lung tissue/cells. **Current research:** In this new data our lab increased *INSR* levels in liver, skeletal muscle and lung to see if acute insulin therapy could be enhanced to further improve AAV transduction in skeletal muscle and liver and to make the insulin treatment effective in lung gene transfer. Our lab also made liver and skeletal muscle less responsive to insulin to determine if this would impact acute insulin therapy to improve transduction. **Results:** Hep3B human liver cells fasted (low glucose/FBS) for 24hrs elevated *INSR* gene expression 5 fold. Co-administrating insulin with AAV2-eGFP showed that insulin

treatment improves AAV transduction 3 fold in Hep3B cells grown under normal conditions and that this increase was enhanced by fasting cells before transduction (5 Fold over non-fasted control cells with no insulin treatment and 1.7 fold over non-fasted cells with insulin). BEAS lung human epithelial cells and A549 lung human alveolar cells were fasted and showed a 6 and 8 fold increase in *INSR* expression respectively compared to non-fasted cells. Insulin treatment in non-fasted BEAS and A549 cells did not improve AAV2-eGFP transduction but in fasted cells co-administration of insulin significantly improved transduction 2.7 fold in BEAS cells and 2.7 fold in A549 cells over controls (non-fasted /no insulin) and 2.3 and 2.6 fold respectively when compared to non-fasted cells with insulin treatment. In mice, fasting for 18hrs resulted in a 7 and 9 fold increase of *INSR* gene expression skeletal muscle and liver respectively. Liver transduction using AAV8-schFIX or skeletal muscle transduction using AAV1-schFIX was done with or without fasting and with or without acute insulin treatment. Acute insulin therapy without fasting improved transduction by 3-5 fold in both liver and skeletal muscle while fasting mice combined with acute insulin therapy improved transduction 5-8 fold in skeletal muscle and 6-9 fold in liver. Decreasing *INSR* mRNA levels in Hep3B cells was also tested. Feeding cells siRNA reduced *INSR* gene expression levels 6 fold and insulin therapy to improve AAV2-eGFP transduction was unsuccessful when the RNA interference treatment was given prior to co-administration of insulin and vector. For in-vivo studies, mice were put in a 28 day hyperinsulemic state using embedded osmotic pumps to deliver insulin. These mice were less responsive to insulin challenges (determined by blood glucose) and biopsies showed 3 and 6 fold decrease in *INSR* mRNA levels in liver and muscle respectively. In mice given the 28 day insulin pre-treatment, acute insulin therapy to improve transduction was ineffective at improving skeletal muscle transduction using AAV1-schFIX and less successful at increasing transduction associated with liver directed delivery of AAV8-schFIX. These data were compared to control mice only given saline for 28 days. For all mice studies, transduction was assessed by measuring systemic hFIX (human factor X) levels for 56 days. **Conclusions:** Our results show that decreasing *INSR* gene expression has a negative impact on acute insulin therapy to improve AAV transduction to skeletal muscle and liver while increasing *INSR* gene expression has a positive effect. Furthermore, strategies to increase *INSR* levels in lung cells promotes effective insulin treatment that improves transduction.

325. rAAV-8 Production by Using HEK293 Derivatives, Xeno-Free Media and Flow Electroporation-Based Transfection

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Background: In accordance with expanded use of recombinant adeno-associated virus (rAAV) vectors by ongoing clinical trials and commercialization, their scalable manufacturing under xeno-free condition is recommended to reduce the risk of unexpected troubles in the future. In this study, we tried rAAV production by helper-free system using derivative cell lines of HEK293, chemically-defined protein-free media (CD293, FreeStyle 293, FreeStyle F17, Expi 293), and cationic polymer or flow electroporation mediated transfection.

Methods: Suspension-adapted 293H and 293.2sus cells were cultured respectively in CD293 and FreeStyle 293 medium during both cell propagation and rAAV production. Adherent 293EB and 293T cells were cultured respectively with FreeStyle F17 and Expi 293 medium from at least one week before transfection to produce rAAV. Equal amount of a proviral plasmid, a AAV helper plasmid, and an adenovirus helper plasmid were transfected into cells simultaneously by polyethylenimine (PEI) or by electroporation with MaxCyte STX (MaxCyte, Inc.). For PEI transfection, the DNA/PEI ratio of 1:2 (w/w) was used for polyplex formation. Suspended cells in each medium containing polyplex were agitated at 166 rpm for 4 hrs. For electroporation, suspended cells in electroporation buffer containing 400 μg/ml of plasmids at 1.0 x 10^8 cells/ml were treated with MaxCyte STX. After electroporation, the cells were transferred to empty culture dish, and kept at 37 degrees C for 30 minutes. After the transfection, cells were suspended to sufficient volume of each medium for vector production. The supernatants and cells containing rAAV were harvested at 72 to 120 hrs after the transfection, and the genome copies of rAAV were measured by qPCR analysis. A part of harvested supernatant or sonicated cell lysate containing produced rAAV was filtrated, and added to cell culture, and the infectivity was examined.

Results: The cells transfected with flow electroporation successfully produced rAAV even if under the medium which is not recommended for transfection with cation-DNA complex. Adherent derivative cell line of HEK293 could be cultured in suspension by immediate change of culture medium to either FreeStyle F17 or Expi 293. Genome copies of rAAV produced by PEI transfection were higher than those of electroporation by preset protocol for HEK293. The rAAVs produced under xeno-free conditions successfully transduced target cells in vitro.

Conclusion: rAAV was successfully produced with completely xeno-free condition and with transfection by both cationic polymer and flow electroporation, although further efforts will be required to improve the vector production efficacy.

326. Formulation Buffer Development for AAV8 Gene Therapy

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Background A major drawback of adeno associated virus serotype 8 (AAV8) gene therapy is the virus' lack of stability, generation of particulates during storage, and adsorption of viral particles on surfaces of contact materials. We developed a liquid formulation to minimize the challenges described above and enable storage of the AAV8 gene therapy material for an extended period when frozen.

Aim To develop a formulation to stabilize the AAV8 gene therapy vector, and prevent particulate formation and adsorption onto contact materials during processing and storage.

Methods A set of formulation matrices were screened for their ability to stabilize the AAV8 vector. Medium to long-term stability studies were established at +5, -20, and <-60 °C for 5 months**.** Appearance, viral infectivity (with in vitro, in vivo biopotency and FIX-qPCR assays), and total particle titer (using rAAV8 particle ELISA) were analyzed. Chemical and physical degradation pathways were also assessed using SDS-PAGE and Size Exclusion Chromatography (SEC) techniques, respectively. Finally, adsorption with contact materials, encountered during processing and storage, was evaluated. Toxicological/clinical input, implementation of the formulation into the downstream process, and IP status were taken into account in selecting the formulation.

Results One of the formulations tested stabilized the AAV based product for at least 5 months when stored at -20 and <-60°C, and for at least 4 months at +5°C. No visible particles were observed during storage. No significant loss of infectivity or total particle titer was observed during the study. SDS-PAGE and SEC techniques did not show significant chemical or physical degradation. Adsorption studies did not reveal any loss of the AAV active with contact materials.

Conclusion The selected formulation has been incorporated into the AAV8 vector manufacturing process.

327. Recombinant AAV8 Vector Purified by Rapid Ultrafiltration Method Safely and Efficiently Transduces Liver

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It is generally assumed that viral vectors have to be extensively purified for use in medical research, in order to eliminate impurities related to the production process that might cause toxicity and to separate and concentrate biologically active virus particles. For recombinant adeno-associated virus (rAAV) vectors this is typically accomplished using density gradient-based methods, which are tedious and require specialized ultracentrifugation equipment. We have recently devised a simple and rapid ultrafiltration approach that permits simultaneous concentration and partial purification of virus from producer cells and media supernatant. Here we show that systemic administration of rAAV8 vectors prepared by this rapid purification (RP) method is safe and efficiently transduces the liver. Across a range of doses, delivery of RP-based rAAV8-CMV-eGFP vector induced enhanced green fluorescent protein (eGFP) expression in liver that was similar to that obtained from a conventionally iodixanol-purified (IP) vector. No liver inflammation was detected in animals that had received either the IP or the RP-based vectors, revealing that residual impurities in the viral vector preparation are not deleterious to the host. Finally, both IP and RP-based vectors induced similar levels of antiviral neutralizing antibody responses. Together, these data demonstrate that partially purified RP-rAAV vectors are a new and useful tool for in vivo research studies. The speed, versatility, and cost-effectiveness of the RP method and lack of need for specialized equipment will enable more extensive use of rAAV vectors in liver gene transfer studies.

328. Stabilization and Storage of AAV Over Multiple Heating and Freezing Cycles

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Aggregation is a commonly observed problem associated with preparations of purified viral vectors, such as adeno-associated virus (AAV) vectors. We sought to devise a formulation of non-toxic ingredients suitable for in vitro and in vivo use, to help stabilize AAV over multiple freeze thaw cycles and/or prolonged periods of storage. The biological activity of purified AAV within the storage stabilizer solution was compared to a mock control of purified AAV in elution buffer after 5+ cycles of repeated heating and freezing.

Cancer-Immunotherapy, Cancer Vaccines II

329. Chimeric Antigen Receptors (CARs) Interact *in cis* with Key T-Cell Proteins, Affecting TCR and Cytokine Signaling, in an Antigen-Independent Fashion

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Adoptive transfer of chimeric antigen receptor (CAR)-T cells is expected to become the first line of treatment for multiple malignancies, following the enormous success of anti-CD19 therapies. However, their mechanism of action is not fully understood, and clear guidelines for the design of safe and efficient receptors are missing.

In order to elucidate the molecular events triggered as a consequence of CAR expression in T cells, we conducted a systematic characterization of the CAR protein 'interactome' using co-immunoprecipitation followed by liquid chromatography tandem mass spectrometry (LC-MS/MS)-based protein identification and bioinformatics analyses. We identified 251 candidate *cis* binding partners of either a second generation or a third generation CAR, targeting prostate stem cell antigen (PSCA). This group was enriched in molecules involved in TCR and cytokine signaling, co-stimulation, metabolism, and RNA binding, among others. Technical validation of these results was conducted using Western blot and/or flow cytometry. Notably, interleukin 2 (IL-2) receptors were identified as CAR binding partners, and their expression was increased in CAR-T cells, in comparison with mock-transduced T

cells. This interaction was associated with increased activation of JAK/STAT pathways in CAR-T cells, suggesting increased sensitivity to IL-2. We then focused on proteins that displayed differential interaction between the second and third generation CARs, observing that second generation CARs interacted more avidly with the endogenous CD3zeta and CD28 than third generation CAR. This interaction was associated with spontaneous phosphorylation of CD3zeta, in absence of antigen recognition, and superior *in vivo* efficacy. Upon antigen binding, TCR signaling was significantly stronger in CAR-T cells expressing second generation CARs, as evidenced by global phosphoproteomic analysis of CAR-T cells co-cultured with pancreatic cancer cells. Differential binding to endogenous CD3zeta was independent of the choice of co-stimulatory domains, but rather depended on the length of the intracytoplasmic tails.

In conclusion, CARs interact with multiple endogenous molecules in steady-state T cells, leading to perturbation of multiple pathways that are relevant for tonic signaling and, potentially, for exhaustion and overall antitumor efficacy. We have developed tools for global, systematic analysis of CAR interactome and signaling, and we are currently implementing the results of this study in the rational design of enhanced antigen receptors.

330. Mixed Signals: Alteration of CD28 Signaling Motifs Improves Anti-Tumor Efficacy and Persistence of CAR T Cells

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Second-generation chimeric antigen receptors (CARs) have incorporated the co-stimulatory domain of either CD28 or 4-1BB, a choice that influences clinical options. While both CAR formulations have induced clinical remissions in patients with relapsed/refractory B-cell malignancies, differences are observed in the persistence of the engineered cell products and can dictate the necessity of a subsequent bone marrow transplant. CAR T cells co-stimulated by 4-1BB exert a long-lived persistence in patients; T cells demonstrate more central memory formation and a characteristic metabolic profile, including increased spare respiratory capacity. CAR T cells co-stimulated by CD28 are no longer detectable in patients after 90 days; these cells are characterized by effector cell formation and increased glycolysis. For solid tumor treatment, CAR T cell persistence may be a necessary requirement to induce clinical remissions. We sought to investigate CD28 signaling within the CAR through mutation of important residues and motifs in an effort to improve persistence. In vitro stimulation of CAR T cells through antigen-coated magnetic beads revealed that CD28 co-stimulated cells exert a rapid signaling response characterized by intense AKT and MAPK signaling as well as a robust calcium flux amplitude and duration. 4-1BB co-stimulation led to a delayed signaling response, increased NFkB signaling, and reduced calcium flux. However, when the CD28 co-stimulatory domain is mutated to decrease PI3K activity or Grb-2 interaction, signaling responses are delayed and calcium flux is reduced, more similar to 4-1BB activity. Interestingly, certain mutations within the CD28 costimulatory domain increased the spare respiratory capacity of the CAR T cells. We investigated the ability of mutant CAR T cells to reduce tumor burden in xenograft mice and assessed engineered cell persistence. We discovered one mutation that outperformed conventional CD28-costimulated CAR T cells in both tumor rejection and cell persistence. Therefore, we suggest that CD28-costimulated CAR T cells can be retooled to a more effective and long-lived phenotype in patients through mutation of CD28 signaling motifs.

331. CAR T Cells Combined With PD1 Blockade Promote Long-Term Efficacy in a Mouse Model for High Grade Gliomas

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New treatments for high grade gliomas (HGGs) are urgently needed. Due to their anatomical location and infiltrative growth complete surgical resection is rarely achieved and hence - despite adjuvant treatment with chemoradiation - tumour recurrence is virtually inevitable. Chimeric antigen receptor (CAR) T cell therapy has achieved durable responses in chemorefractory $CD19^+$ haematological malignancies. However, treatment of solid tumours is proving more difficult. Several studies suggest that efficacy in this setting requires additional co-stimulation. Combination with PD1 blockade has shown encouraging results, enabling CAR T cells to overcome an immunosuppressive microenvironment and deliver potent responses. We used Epidermal Growth Factor Receptor variant III (EGFRvIII) - present in 30% of gliomas - as model antigen to develop CAR T cell therapy for HGGs. In order to study the interplay between CAR T cells, the tumour and the endogenous immune system, we chose a syngeneic mouse model. The mouse glioma cell line GL261, modified to express EGFRvIII, was used to establish orthotopic tumours and evaluate efficacy of mouse T cells expressing a 2^{nd} generation CAR (CD28-CD3ζ) with the scFv of the αEGFRvIII antibody MR1.1. We incorporated CD34 as marker gene, while luciferase was introduced for *in vivo* tracking. After validation of function and specificity *in vitro*, we tested efficacy of EGFRvIII CAR-T cells *in vivo*. GL261_EGFRvIII were intracranially implanted in C57Bl6 mice. Following confirmation of engraftment with magnetic resonance imaging (MRI) 10 days post implantation, mice received total body irradiation and systemic infusion of $5x10^6$ CAR T cells. Bioluminescence imaging demonstrated that CAR T cells migrated to the tumour and accumulated over time in an antigen-dependent manner. Specific infiltration was confirmed both by FACS and CD34 immunohistochemistry (IHC) CD34. Serial MRI demonstrated that CAR T cells delayed tumour growth and increased survival ($p \leq 0.01$). However, tumours were not consistently eradicated. CD34 IHC showed loss of CAR T cells within the tumour at sacrifice, suggesting lack of long term persistence. This was not due to loss of antigen expression, as treated tumours were still $EGFRvIII^+$, FACS analysis at 9 days post infusion (dpi) showed that not only CAR T cells were active - Granzyme B (GzmB) 91%±5, Ki67 68.2%±29 - but their administration resulted in an overall activation of the endogenous

compartment. Conversely, at 17 dpi, CAR T cells exhibited a marked decrease in GzmB (64%±12) and Ki67 (46%±20) expression. As CAR T cells were positive for PD1 and LAG3, we hypothesised that this may be due to exhaustion. We therefore tested whether PD1 blockade could restore their function. Mice received 4 doses of the PD1-blocking antibody RMP1-14. Both MRI and H&E showed that combination therapy promoted complete clearance of tumours and long term survival. Characterisation of tumour infiltrating T cells with and without αPD1 administration revealed no significant differences in phenotype of both transferred and endogenous cells. Nonetheless, at 17 dpi - when tumour eradication was almost complete - mice receiving combination therapy exhibited higher infiltration (counts/mm^3) of CAR T cells, compared to mice receiving CAR T cells only. This finding was confirmed by CD34 IHC at a later time point, soon after tumour eradication (30dpi). This suggests that PD1 blockade can promote persistence of T cells *in situ*. We conclude that, for CAR T cell therapy to be an effective treatment for HGGs, combination with PD1 blockade may be required to promote a potent and persistent response.

332. ICOS-Based Third Generation CARs for the Treatment of Solid Tumors

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We have previously demonstrated that the incorporation of the ICOS costimulatory domain in a chimeric antigen receptor (CAR) is critical for the function and persistence of CD4+ T cells while 4-1BB costimulation enhanced the persistence of CD8+ T cells. We also established that a combination of ICOS and 4-1BB in a third generation CAR targeting mesothelin (SS1-CAR) has enhanced antitumor effect with increased in vivo persistence in a pancreatic cancer xenograft mouse model. This enhanced antitumor effect was observed despite significantly lower levels of CAR expression in administered T cells. Here we investigated the mechanism behind this enhanced antitumor effect. We hypothesized that reduced CAR expression increases T cell resistance to exhaustion in the presence of continued antigen exposure and thereby improves anti-tumor activity. In order to test this hypothesis, we generated a lentiviral vector with a pGK300 promoter to express SS1-BBz CAR with cell surface levels similar to ICOSBBz expression, which was confirmed by flow cytometry. When administered into animals bearing pancreatic tumors, pGK300-BBz T cells were unable to induce any anti-tumor effect when compared to non-treated animals. Treatment with T cells expressing high levels of the BBz CAR (driven by the EF1a promoter) exhibited transient tumor regression. Despite significantly lower levels of CAR expression than the EF1a-BBz, T cells expressing ICOSBBz under the EF1a promoter were significantly more efficient at controlling tumor growth. These results demonstrate that the superior anti-tumor efficacy of the ICOSBBz CAR is not a consequence of its low expression levels. Next, we sought to investigate whether signaling through two costimulatory domains, including ICOS and 4-1BB, is superior to signaling through either component alone. We hypothesized that if combining ICOS and 4-1BB signaling enhances antitumor effect, a third generation CAR containing both ICOS and 4-1BB but in the reverse order (BBICOSz), in which the 4-1BB domain is positioned proximal to (and the ICOS domain distal to) the plasma membrane, should also show superior antitumor effect when compared to second generation CARs. To test this, NSG mice bearing pancreatic tumors were treated with redirected T cells and overall survival was analyzed. Interestingly, mice receiving ICOSBBz CAR-T cells showed enhanced survival compared to those receiving either BBICOSz or BBz CARs. Whereas less than 40% of the animals from the BBz and BBICOSz groups were alive by day 56, 100% of the animals treated with ICOSBBz were alive at the same time point. Overall these results indicate that the combination of ICOS and 4-1BB in a third generation CAR can be more effective at eliminating large pancreatic tumors than either 4-1BB or ICOS alone. However this effect is only observed when ICOS is proximal to the cell membrane, which suggests membrane proximity is a critical consideration for third generation CAR development.

333. Effective and Reversible Control of Anti-Tumor Activity *In Vivo* with a Drug-Regulated CAR T Cell Platform (DARIC)

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Redirecting T cells against tumors by introducing antigen-specific chimeric antigen receptors (CAR) has shown promising clinical results as a potential treatment strategy for certain cancers. However, traditional CARs are constitutively active, resulting in two potential liabilities - first, the long term-toxicity due to persistent loss of all target cells including normal tissues/cells that express the target antigen (off-tumor, on target activity) and second, the potential for persistent T cell activation to drive either T cell exhaustion or excessive cytokine release. While "off switches" based on suicide cassettes or other depleting cell approaches are being explored, such systems by definition result in the elimination of the therapeutic cells. Here we have developed a novel drug-regulated CAR-based antigen targeting approach termed Dimerizing Agent Regulated Immune-receptor Complex (DARIC) that aims to: i) minimize the long-term toxicity of CAR T treatment; ii) lessen persistent T cell activation; and iii) be amenable to multiplex antigen targeting. The DARIC platform separates the antigen recognition and signaling functions of a CAR into two distinct polypeptides that are further engineered to contain the FKPB12 and FRB small-molecule regulated dimerization domains. In the absence of the dimerizing drug (e.g. rapamycin or the non-immunosuppressive rapalog AP21967) the DARIC system lacks signaling activity. However, the addition of dimerizing agent drives the interaction of the two DARIC subunits, fully restoring CAR function. Using CD19 as a model system, we show that treatment of CD19-DARIC+ T cells with rapamycin or AP21967 results in equivalent cytotoxicity, cytokine production and proliferation compared to a standard CD19-targeting CAR. Importantly, CD19-DARIC T cells were activated by low (10pM) levels of rapamycin and exhibited a higher antigen sensitivity than standard CD19-CAR T cells *in vitro*. In an aggressive CD19+Nalm-6 xenograft tumor mouse model, CD19-DARIC T cells did not exhibit anti-tumor activity in the absence of

dimerizing agent. However, CD19-DARIC treated mice that received either rapamycin or AP21967 showed an equivalent level of tumor control compared to standard CD19-CAR treated animals despite the use of comparatively low doses of rapamycin (0.1mg/kg). This activity was dependent on the presence of the dimerizing drug, as cessation of drug treatment resulted in the loss of CD19-DARIC T cell activity and the expansion of Nalm-6 tumors cells in the DARIC T cell treated mice, consistent with the ability to switch off CD19-DARIC T cells *in vivo* by withdrawing drug. Taken together, these results highlight the potential of the DARIC platform to facilitate the regulation of CAR T cell function *in vivo*.

334. Combination of Oral WT1 Cancer Vaccine and Anti-PD-1 Antibody Induced the Synergistic Anti-Tumor Effect in Mouse Prostate Cancer Model

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INTRODUCTION: Prostate cancer is the most common cancer in men, and is the sixth leading cause of cancer-related death worldwide. Recently, a dendritic cell-based vaccine, sipuleucel-T®, has been approved by US FDA for the treatment of metastatic castration-resistant prostate cancer (mCRPC), however, it achieved only modest clinical efficacy and its production by culturing autologous dendritic cells takes a lot of efforts and extremely high cost. Previously, we had constructed a recombinant *Bifidobacterium longum* displaying Wilms' tumor 1 (WT1) protein (named *B. longum* 420) as an oral cancer vaccine and demonstrated that oral administrations of *B. longum* 420 could induce the WT1-specific cellular immunity in mice. In addition, about 50% of advanced prostate cancer expresses WT1. In the present study, we investigated the feasibility of combination immunotherapy of *B. longum* 420 and anti-programmed death 1 (PD-1) antibody for the treatment of prostate cancer using mouse tumor model. **METHODS:** We evaluated the anti-tumor effect of *B. longum* 420 and anti-murine PD-1 antibody using murine prostate cancer cells, TRAMP-C2, which expressed WT1, and PD-L1 induced by interferon-γ. Firstly, 2×10^6 of TRAMP-C2 cells were inoculated into mice. After tumor formation, 1×10^9 colony forming units of *B. longum* 420, *B. longum* 2012, which was mock control, and PBS were orally administered into mice 5 days a week over the following weeks. In addition, 100 μg anti-murine PD-1 antibody or isotype control were intraperitoneally injected at day 31, 34, 38, 41 and 45. To analyze the WT1-specific cellular immune responses induced by *B. longum* 420, mice splenocytes were isolated and their cytokine production, cytotoxic activities against TRAMP-C2 were determined. **RESULTS:** Oral administrations of *B. longum* 420 and anti PD-1 antibody significantly inhibited the growth of TRAMP-C2 tumor compared with the group treated with *B. longum* 420 and isotype control treatment at day 56 (82.7±10.3 mm^3 vs 176.2±18.1 mm^3). The combination therapy also significantly improved the overall survival compared with *B. longum* 2012 with anti PD-1 antibody and PBS. The splenocytes cultured with TRAMP-C2, produced significantly higher IFN-γ, IL-2 and TNF-α in *B. longum* 420 group compared to both *B. longum* 2012 and PBS group. *B. longum* 420 also induced significantly higher population of $CD4^+$T and $CD8^+$T cells that were simultaneously producing IFN-γ, IL-2 and TNF-α. MHC-tetramer assay using the representative WT1-$CD8^+$T epitope revealed that *B. longum* 420 induced that epitope-specific CTLs by immunization with whole WT1 protein displayed on the *Bifidobacterium*. Also, CTLs isolated from *B. longum* 420 group elicited significantly higher cytotoxicity against TRAMP-C2 in a effector:target ratio dependent manner *in vitro*. **CONCLUSIONS:** We demonstrated that an immunotherapy using *B. longum* 420 and anti-PD-1 antibody could safely induce the synergistic anti-tumor effect in mouse prostate cancer model. These findings suggested that the combination immunotherapy of oral WT1 cancer vaccine and anti-PD-1 antibody could be applied for the treatment of mCRPC.

335. Tunable CAR T Cell Responses Achieved by the Disruption of Protein-Protein Interactions via the Administration of a Small Molecule

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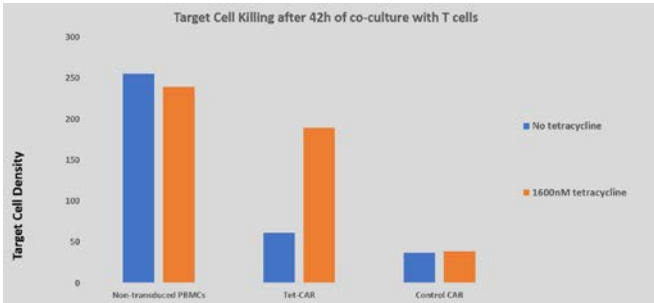
Introduction T cells expressing chimeric antigen receptors (CARs) have shown high level of durable responses in haematological malignancies. However significant side effects, like cytokine release syndrome or neurological side effect were observed in the majority of patients, requiring intensive care treatment and ventilation in as many as 20 percent of treated patients. While side effects were mostly reversible, there were also deaths reported due to side effects. The key driver for side effects is the high level of T cell activity and we hypothesized that temporarily turning the CAR-T cells off might provide a means to manage the side effects and avoid the need for intensive care treatment. To this end, we have designed a CAR with a non-covalently bound intracellular activating domain. Presence of a small molecule widely used in clinical practice displaces the intracellular activating domain, turning the CAR off. Removal or clearance of the small molecule allows the CAR to recouple to its activating domain and render the CAR T cell active again. **Methods and Results** We established a CAR with a soluble intracellular activating domain. The activating domain comprised of CD28 and CD3zeta activation domains and bound via a specific peptide to a tetracycline binding domain fused to the CAR. The CAR is active in the absence of tetracycline or its analogs. Presence of 1600 nM, or less tetracycline displaced the peptide from the tetracycline binding site and turned the CAR-T cell off, as shown by dose dependent loss of cytotoxic activity and loss of cytokine release by the CAR-T cells. Removal of tetracycline then lead to recoupling of the CAR and the CAR-T cells regained cytotoxic activity and expressed cytokines. **Conclusion** We successfully generated a small molecule controlled CAR-T approach that enables fine tuning of CAR T cell activity via the administration

of an approved and readily available small molecule. Importantly, small molecule intervention is only required to reduce T cell activity, rather than requiring continued presence of a small molecule to sustain CAR T cell activity as is needed with rapalog dimerizers.



Cytotoxicity Assay Cytotoxicity of the CAR is determined based on the ablation of the target cells over time in co-cultures with the CAR expressing T cells. Target cells are fluorescent and their expansion is microscopically monitored. Co-cultures of Tet-CAR/control CAR expressing cells with red fluorescent target were set up at an 8:1 effector to target cell ratio. Co-cultures were carried out in the presence or absence of 1600nM tetracycline. The changes in target cell density were determined over a period of 42h.

336. Cancer Immunotherapy Mediated by Combined p19Arf and Interferon-Beta Gene Transfer: Evidence from Vaccine and *In Situ* Gene Therapy Models

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Recent trends for cancer treatment include a variety of strategies that elicit a sustained anti-tumor immune response. We have developed a gene transfer approach that is expected to promote both cell death and immune stimulation due to the activity of p19Arf (a functional partner of p53) and interferon-beta (IFNβ, a pleiotropic cytokine), respectively. Here we explore evidence for cooperation between these players as well as their application as an immunotherapy in mouse models of melanoma and lung carcinoma. *In vitro* studies have revealed that combined p19Arf and IFNβ gene transfer enhances cell death and that only the combination is able to induce a variety of multi-modal, immunogenic cell death markers. We have noted that *in situ* gene therapy of B16F10 tumors (s.c.) with combined, but not individual, p19Arf and IFNβ was associated with elevated levels of cell death (TUNEL) or perinuclear accumulation of LC3β, indicating autophagy. When applied as a prophylactic vaccination in an immune competent model, B16F10 cells treated with the combination unleashed an NK cell mediated rejection of the treated cells and a CD4+ and CD8+ T cell dependent protection against a contralateral tumor challenge. Strikingly, in a therapeutic vaccination model using TM1 melanoma cells, IFNβ treatment alone did not affect tumor growth whereas the p19Arf/IFNβ combination promoted a significant reduction in tumor progression. Exploring a different immunization context, using the LLC (lewis lung carcinoma) model, *in situ* gene therapy in immune competent mice was followed by implantation of fresh, untreated LLC cells. We noted the reduction in challenge tumor progression only when the primary tumor had been treated with the p19Arf/IFNβ combination. In addition, prophylactic vaccination with LLC cells treated with combined, but not individual, gene transfer provided gene transfer was able to slow growth of challenge tumors. Microarray analysis of cDNA derived from tumors treated *in situ* revealed enhancement of chemokines, especially those associated with recruitment of neutrophils. Indeed, elevated numbers of neutrophils were found in the tumors treated with the combination. Functionally, the depletion of granulocytes reversed the benefit of combined gene transfer, indication the participation of this cell population. Work in progress includes association of our immunotherapy approach with chemotherapy and/or checkpoint blockade. We have seen that sub-therapeutic levels of doxorubicin reduce tumor progression only when applied in conjunction with p19Arf and IFNβ. This low level treatment with doxorubicin avoided cardiotoxicity seen with the therapeutic dose. Thus, our gene transfer method may provide a safety advantage as compared to the use of chemotherapies for the induction of immunogenic cell death. In all, p19Arf and IFNβ cooperate to form an effective immunotherapy when applied in prophylactic or therapeutic vaccines or even upon *in situ* gene therapy. Financial support: Sao Paulo Research Foundation, grant (BES, 15/26580-9, 13/25167-5) and fellowships (RFVM, JPPC, AH).

337. Defining an Allogeneic CAR-T Approach by shRNA-Mediated Knockdown of the T-Cell Receptor

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INTRODUCTION: CAR T-Cell therapy is an exciting advancement in the field of oncology that provides the ability to modify a subject's own immune cells to treat their cancer. Although autologous adoptive cell transfer has been successfully employed in the clinic, an allogeneic approach has the potential to significantly streamline the manufacturing process, thus providing more accessible options to patients as well as enhancing safety by reducing the possibility of graft-versus-host-disease from an HLA mismatched donor. The T-Cell Receptor (TCR) is a protein comprised of multiple subunits and functions to activate T-cells by a signal transduction cascade that is initiated upon antigen binding. Thus, restricting or eliminating expression of the endogenous TCR on the modified CAR-T-Cells may help eliminate the ability to recognize major and minor histocompatibility antigens in the recipient. The goal of this study was to assess if the simultaneous expression of multiple short hairpin RNAs that knockdown levels of individual TCR subunits could result in the complete loss of TCR-mediated T-Cell activation.

METHODS: Recombinant DNA expression constructs producing combinations of short hairpin RNA (shRNA) against the various subunits comprising the TCR complex, were transfected into T cells. Cell surface TCR expression was analyzed by FACS. Following CD3 activation or co-culture with B-cells, T-Cell activation was quantified by measuring the levels of IL-2 by ELISA and QPCR.

RESULTS: As even modestly low levels of TCR activity might be detrimental in an allogeneic approach, a strategy was employed to completely abrogate TCR activity. When expressed individually, each shRNA inhibited protein and mRNA expression of it's cognate TCR subunit by up to 93% of the endogenous levels. However, upon simultaneous expression of the shRNAs against the different subunits from the same vector, we observed a near complete depletion of the TCR

complex from the cell surface (>99%) as measured by FACS analyses. Furthermore, TCR functionality was inhibited when treated cells were stimulated with either CD3 or in B cell co-cultures with Staphylococcal enterotoxins. IL-2 secretion was inhibited to undetectable levels by ELISA by the multi-shRNA treatment and >98% by qPCR.

CONCLUSIONS: Although the level of individual knockdown of any one of the components of TCR never exceeded 93%, simultaneous knockdown of several TCR subunits was sufficient to abrograte surface TCR expression and downstream activation suggesting that disruption of stoichiometric expression levels of the subunits was sufficient to prevent TCR formation. Given the small size of each shRNA expression cassette, the packaging capacity required for three shRNAs (<2 Kb) permits co-expression from the same lentiviral vector as the CAR. Altogether, these data point to a viable strategy towards generating a single vector approach for the production of allogeneic T-Cells for immunotherapies against certain cancers.

338. Oncolytic Adenovirus Coexpressing Interleukin-12 and shVEGF Restores Antitumor Immune Function and Enhances Antitumor Efficacy

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Tumor microenvironment is extremely immunosuppressive, preventing efficient induction of antitumor immunity. To overcome tumor-mediated immunosuppression and enhance the potency of immunogene therapy, oncolytic adenovirus (Ad) expressing interleukin (IL)-12 and vascular endothelial growth factor (VEGF)-specific short hairpin ribonucleic acid (shVEGF; RdB/IL12/shVEGF) was generated. Intratumoral injection of RdB/IL12/shVEGF induced a strong antitumor effect in an immune competent B16-F10 melanoma model. RdB/IL12/shVEGF restored immune surveillance function in tumor tissues and actively recruited immune cells by elevating the expression levels of IL-12 and interferon-γ. RdB/IL12/shVEGF efficiently suppressed expression of immunosuppressive VEGF, resulting in restoration of the antitumor immune response and prevention of thymic atrophy. *In situ* delivery of RdB/IL12/shVEGF to tumor tissues resulted in massive infiltration of differentiated $CD4^+$ T cells, $CD8^+$ T cells, natural killer cells, and dendritic cells to tissues surrounding the necrotic region of tumor. Furthermore, RdB/IL12/shVEGF induced a potent tumor-specific T helper type 1 immune response, implying that attenuation of the immunosuppressive environment mediated by downregulation of VEGF can significantly enhance immune stimulatory functions in the tumor milieu. Collectively, these findings indicate the potential of inducing and restoring potent antitumor immunity using intratumorally administered oncolytic Ad co-expressing IL-12 and shVEGF.

339. A Cancer Stem Cell Vaccine Engineered to Express Interleukin-15 and its Receptor Induces T Cell Proliferation

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Introduction. Interleukin-15 (IL-15), a member of the 4-α-helix bundle family of cytokines is a powerful activator and inducer of maturation of both NK and CD8+ cytolytic T cells. It also activates and expands CD8+ T memory cell populations without stimulating immunosuppressive CD4+CD25+ T-regulatory cells. This suggests that IL-15 may be useful as an immunotherapy for the treatment of cancer. In a phase I clinical trial, infusions of recombinant human IL-15 achieved tumor regressions; however, treatment was associated with a systemic inflammatory response syndrome.[†] In an effort to enhance antitumor activity and reduce systemic side effects, we studied an approach using a vaccine enriched for cancer stem cells (CSCs) genetically altered to express murine (m) IL-15 and its receptor (mIL-15Ralpha). **Materials & Methods.** A series of lentiviral vectors expressing the wild type (*wt*) or optimized (*opt*) cDNA sequences for murine mIL-15 and/or mIL-15Ralpha under the control of the human EF-1 promoter with puromycin resistance (*puroR*) as a selective marker were generated and used to transduce TC1 mouse lung cancer cells. The TC1 cells were cultured under low serum conditions to generate tumor spheroids enriched for CSCs. **Results.** On RT-PCR analysis, the various transduced TC1 cells demonstrated the expected mRNA transcripts. However, cells transduced with constructs expressing mIL-15^{wt} did not show consistent expression of mIL-15 protein. On flow cytometry the cells transduced with mIL15Ralpha (*wt* or *opt*) alone or in combination with mIL-15^{opt} showed surface expression of both mIL-15Ralpha and mIL-15, while cells transduced with mIL-15^{opt} construct did not. When co-cultured with the transduced TC1 spheroids or incubated with supernatants obtained from these cells, CTLL-2 murine T cells demonstrated sustained proliferation indicating that the cloned cytokine and receptor were expressed and functional. The vector demonstrating the greatest stimulation of CTLL-2 cells expressed both the mIL-$15Ralpha^{wt}$ and mIL-15^{opt} sequences in this order. **Conclusion.** Mouse lung CSCs expressing wt mIL-15Ralpha and an optimized mIL-15 stimulated the proliferation of mouse T cells suggesting the ability to enhance cellular immune responses. *In vivo* mouse tumor vaccination studies are in progress.

[†]Conlon KC, Lugli E, Rosenberg SA, *et al.* (2015) *J. Clin Oncol.* 33:74-82.

340. Immunological Synapse Predicts Effectiveness of Chimeric Antigen Receptor (CAR) T Cells

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Adoptive cell-based therapy using chimeric antigen receptors (CAR)-modified T cells has the potential to improve the overall survival of patients with malignancies by enhancing the effectiveness of T cells. Precisely predicting the effectiveness of a variety of CAR T cells represents one of today's key unsolved problems in immunotherapy. Optimal function of T cells depends on the quality of the immunological synapse. However, key differences in the immunological synapse formed by effective versus less-effective CAR T cells with their susceptible tumor cells remain unclear. Here, we predict the effectiveness of CAR T cells by evaluating the quality of the CAR T synapse, by quantitation of F-actin, central clustering of tumor antigen, polarization of lytic granule, and distribution of key signaling molecules within immunological synapses. Long-term killing capability, but not secretion of conventional cytokines or standard four-hour cytotoxicity, correlates positively with the quality of the immunological synapse in two different CAR T cells that share identical antigen specificity. The data suggest that the quality of the synapse correlates with performance of CAR T cells in vitro. Therefore, we propose that the quality of the synapse predicts the effectiveness of CAR T cells, which provides a novel strategy to direct CAR T therapy.

341. IL13Ra2-Specific Chimeric Antigen Receptor T Cells Directed to Glioblastoma Suppress Tumor Growth in a Mouse Glioma Model

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Glioblastoma multiforme (GBM) is the most challenging brain tumor due to tumor recurrence. There is currently no effective treatment. Therefore, new therapeutic strategies are urgently needed to stop tumor recurrence and improve patient survival. CAR therapies hold promise in the treatment of GBM without the usual toxicities associated with cytotoxic regimens, and have the potential to improve both survival and quality of life. We exploited interleukin 13 receptor a2 (IL13Ra2) as a GBM-specific tumor antigen due to its frequent overexpression on a majority of GBM but not expressed on normal brain tissues. We have developed and preclinically tested *novel* IL13Rα2-specific CAR T cells for clinical trials. We have proved that the systemic administration of human IL13Rα2-specific CAR T cells have antitumor activity in a mouce glioma xenograft model, mice with day 7 tumors received 5×10^6 IL13Rα2-specific CAR T cells by tail vein injection. Significant increase in survival was observed. These data provide evidence for an effective therapy of the treatment of GBM.

Cell Therapies II (Tissue Engineering)

342. Notch Signaling is Involved in Regulation of Cardiac Stem Cells Behavior in Cell Sheet After Epicardial Implantation

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Cardiac progenitor cells (CPC) based cell sheet transplantation is emerging as a promising method to repair heart injuries. CPC contributes recovery of heart injuries by multilineage differentiation, paracrine secretion and anti-inflammatory effects. However, signaling pathways which regulates cell suvirval and function after transplantation are not investigated in detail. The Notch pathway is a main regulators of the stem cell behavior by interactions between adjacent cells.Here we investigated the role of Notch pathway in the regulation of CPC function in vitro and after epicardial implantation in scaffold free cell sheet.After coronary artery ligation in rats syngeneic c-kit+Lin CPC marked with CM-DIL were grafted by epicardial placement of cell sheet generated using temperature-responsive dishes. Cell sheets integration, neovascularization, Notch signalling activation state, proliferation and differentiation were assessed by immunofluorescence analysis of myocardial frozen sections harvested 14 days after transplantation. For Notch signalling activation NICD overexpression and cultivation of CPC on Jagged 1-coated dishes were used.Histological analyses revealed that CPC sheet grafts produced thick, well vascularized tissues on the epicardial surface of the heart. Part of transplanted CPC migrated into myocardium, showed signs of Notch signaling activation (NICD in nucleus) and differentiation to cardiomyocytes and endothelial cells. Cultivation of CPC in vitro on dishes coated with Jagged 1 (Notch ligand) released NICD and activated expression of Notch target genes (Hes and Hey). Activation of Notch signaling upregulated expression of vascular cell transcription factors in CPCs and γ-secretase inhibitor prevented Notch signaling activation and CPCs commitment to endothelial lineage. Notch activation in CPC increased their ability for tube formation in Matrigel angiogenesis assay. These findings suggest that targeted modulation of Notch1 signalling may be useful for upregulation of cardiac progenitor cell sheets functionality and vacularization.

343. Next Generation Stem Cell-Cardiomyocyte Interactions: Focusing on the Paracrine Effector Approach

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Stem cell research for treating or curing ischemic heart disease has, to date, culminated in three basic approaches: the use of induced pluripotent stem cell (iPSC) technology, reprogramming cardiac

fibroblasts, and cardiovascular progenitor cell regeneration. As each approach has been shown to have its advantages and disadvantages, the challenge has been to minimize the disadvantages while exploiting the advantages. Using human germline pluripotent stem cells (hgPSCs) along with a modified version of a relatively novel cell culture methodology (conditional expansion), it was possible to emphasize the advantages of all three approaches. We consistently find that unipotent germline stem cells when removed from their niche and cultured in the correct medium express, endogenously, Yamanaka factors, inducing them to become hGPSCs and expansion of their potential to form cell types from all three germ layers. Upon differentiating hgPSCs into cardiac lineages, our data consistently shows that they not only express cardiac genes, but also express cardiac-promoting paracrine factors. Taking these data a step further, we found that hgPSC-derived cardiac cells can fuse with cardiac tissue in vivo. Note, while the work presented here was based on testes-derived hGPSCs, data from other laboratories show that ovaries contain very similar types of stem cells that can give rise to hgPSCs. As a result, hgPSCs should be considered for eventual use in patients, male or female, with ischemic heart disease.

344. A Comparison of BMP2 Delivery by Gene Therapy and Coacervate for Promoting Human Muscle-Derived Stem Cell-Mediated Cartilage Repair

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Human muscle-derived stem cells (hMDSCs) have been shown to be multipotent in vitro and repair bone defect in vivo when transduced with lenti-BMP2. BMP2 transduced hMDSCs can repair critical-sized bone defects as efficiently as human bone marrow-derived stem cells. However, whether hMDSCs have the ability to repair articular cartilage in vivo is unknown. Coacervate is a complex biomaterial that can deliver bioactive growth factors for cardiac and bone repair. In this study, we compared gene therapy and coacervate to deliver BMP2 for promoting hMDSC-mediated cartilage repair. **Material and methods:** 1. Lenti-GFP and Lenti-BMP2/GFP with CMV promoter were constructed. 2. hMDSCs were isolated from skeletal muscle via the preplate technique. hMDSCs were transduced with lenti-GFP and Lenti-BMP2/GFP virus, expanded and sorted via FACS based on GFP fluorescence. 3. Coacervate was prepared according to literature. 4. In vitro chondrogenesis was performed for Lenti-GFP or Lenti-BMP2/GFP transduced hMDSCs using 3D pellet culture. Alcian blue and col2A1 staining were performed .5. In vivo cartilage repair using MIA induced Model. Nude rats were divided into 5 groups (1). PBS +coacervate; (2).BMP2+PBS+hMDSC-LGFP;(3). hMDSC-LBMP2/GFP ;(4). BMP2+coacervate+hMDSC-LGFP(5). BMP2+sFlt1+coacervate+hMDSC-LGFP. Rats were injected with monoidonic acetate(MIA) in the right knee and the left knee was used as control. Two weeks later, rats were injected with different combinations of cells/growth factors as stated above. Rats were sacrificed at 12 weeks, and both the injured and non-injured knees were harvested. MicroCT scanning and histology were performed to analyze cartilage repair in each group. **Results**:1. In vitro chondrogenic pellet culture indicated that hMDSC-LBMP2/GFP transduced cells are more chondrogenic than hMDSC-LGFP by alcian blue positive quantification and Col2A1 staining. 2. In vivo study: (1) MicroCT 3D images showed that non injured knee joints have a smooth surface. The injured knees have different extent of knee joint cartilage erosion as the subchondral bone were obvious in trochlear groove and femoral and tibial condyles. Quantification of knee joint gap difference(non-injured knee minus injured knee) indicated hMDSC-LBMP2/GFP group and BMP2+sFlt1+coacervate+hMDSC-LGFP groups showed significantly smaller gap differences compared to PBS+coacervate group. (2). Alcian blue staining and histology grading indicated all cell injected groups showed better cartilage repair than the PBS+coacverate group. BMP2+sFlt1+coacervate+hMDSC-LGFP group showed the best cartilage repair as demonstrated by histology scores.(3). H&E staining demonstrated that joint surface cartilage are significantly better in all the cells injections group than PBS+coacervate group . GFP staining showed that a few GFP+ cells are found in the regenerated cartilage of knee joints.**Conclusion**: We found lenti-BMP2/GFP-transduced hMDSCs have enhanced chondrogenesis compared to hMDSC-LGFP-transduced cells in vitro. The delivery of BMP2 together with hMDSCs significantly improved cartilage repair. Both delivery of BMP2 with gene therapy and coacervate biomaterials enhanced hMDSC-mediated cartilage repair. Blocking angiogenesis by co-delivery of BMP2, sFlt1, and hMDSC-LGFP achieved the best results for cartilage repair. Endogenous cells recruited by BMP2 contributed more to the cartilage repair than the transplanted cells.

345. *In Vivo* Reprogramming to a Transient Pluripotent-Like State Enhances Regeneration of Injured Skeletal Muscle

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Somatic cells can be de-differentiated towards pluripotency *in vivo* by ectopic expression of defined reprogramming transcription factors (*Oct3/4*, *Sox2*, *Klf4* and *cMyc*, known as OSKM). Sustained expression of such cues triggers tumorigenesis, but strategies relying on transient *in vivo* reprogramming induce pluripotency and proliferation only temporarily, without teratoma formation. Here, we sought to achieve transient reprogramming within mouse skeletal muscle with a localized injection of naked plasmid DNA (pDNA) encoding OSKM. We hypothesized that such approach would enhance the regeneration capability of the tissue after a severe and clinically-relevant injury. A single intramuscular administration of OSKM pDNA rapidly triggered the expression of pluripotency genes (*Nanog*, *Ecat1*, *Rex1*) and a marker of myogenic progenitors (*Pax3*) in the healthy gastrocnemius muscle of various mouse strains, but significant levels were only maintained for 2-4 days. In agreement,

distinct clusters of mononucleated cells expressing pluripotency and early-myogenesis markers appeared among the myofibers soon after injection, and proliferated only transiently. While no dysplastic lesions or teratomas were found, morphometric analysis suggested that the reprogrammed cells successfully re-integrated into the tissue. *Nanog* was also significantly upregulated in the gastrocnemius when OSKM pDNA was administered 7 days after surgical laceration of its medial head. Regeneration was enhanced in reprogrammed tissues, as shown by rapid appearance of centronucleated myofibers and significantly reduced fibrosis compared to untreated controls. While further studies are warranted to achieve optimal functional regeneration, these results demonstrate the potential of transient *in vivo* reprogramming as a novel gene therapy strategy for the treatment of clinically-relevant muscle injuries via generation of transiently-proliferative, pluripotent-like intermediate cells.

346. Muscle Derived Stem Cells Naturally Aging Driven by Cell Cycle Regulation

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The duration of G1 phase in the cell cycle gives stem cells more or fewer chances to be induced either extrinsically or intrinsically. Some cyclin-dependent kinase inhibitors (CDKIs: p18, p16, p21) have been identified as being critical stem regulators of cell fate. However, it is still not entirely clear how these CDKIs perform systemically at different status or different lineages of stem cells during naturally aging. **We hypothesize that** the early onset of aging underlies the dynamic change of cell cycle regulation. We profiled the differences of multilineage differentiation potentials between young and aged MDSCs. We analyzed the relationships between the expression of CDKIs and their roles in the loss of multi-lineage differentiation capacities of MDSCs with increasing age. **METHODS:** A. Isolation of young (3 weeks' old) and aged MDSCs (2 years' old) using a modified preplate techniques. B. Flow cytometry detected cell cycle for both young and old MDSCs. C. Multipotent differentiation including myogenesis, chondrogenesis, adipogenesis, and osteogenesis. D. BMP2/GFP transduction: young and old murine MDSCs were transduced with retro-BMP2/GFP and FACS with GFP. E. Osteogenesis was conducted using monolayer culture in osteogenic medium, and osteogenic differentiation was performed using quantitative-PCR. F. In vivo bone regeneration using young and old BMP2/GFP transduced MDSCs in a critical-sized calvarial bone defect model. Bone regeneration was performed using microCT biweekly for six weeks. **RESULTS:** We found that old MDSCs had more G0/1 phase cells and fewer G2/M cells. Interestingly, we found that old MDSCs expressed the significantly higher level of p16, p21 and lower level of p18, compared to young MDSCs. Furthermore, we found that the myogenic, the chondrogenic and the adipogenic differentiation were also significantly lower for old MDSCs than young MDSCs. However, after BMP2/GFP transduction, p18 and p21 became similar between young and old MDSCs, although p16 expression was still higher in old MDSCs. In vitro, osteogenesis was also getting similar between young and old MDSCs regarding RUNX2, osterix, and collagen-I. ALP gene expressed highly at the earlier time point in old MDSCs but was exceeded at the later time point by young MDSCs. Furthermore, BMP2/GFP transduced old MDSCs regenerated less bone than young BMP2/GFP transduced MDSCs. However, interestingly, the difference was more striking at earlier time points after cells transplantation as old MDSCs group caught up with young MDSCs group in bone volume at a later time point.**Conclusion:** In this study, we found that old MDSCs had cells cycle changes significantly with more cells in G0/1 phase. Old MDSCs demonstrated impaired multipotent differentiations in vitro and bone regeneration in vivo. BMP2 transduction could regulate the cell cycle change in old MDSCs. Therefore cell cycle regulation may be one of the strategies to reverse the age-related stem cells dysfunction.

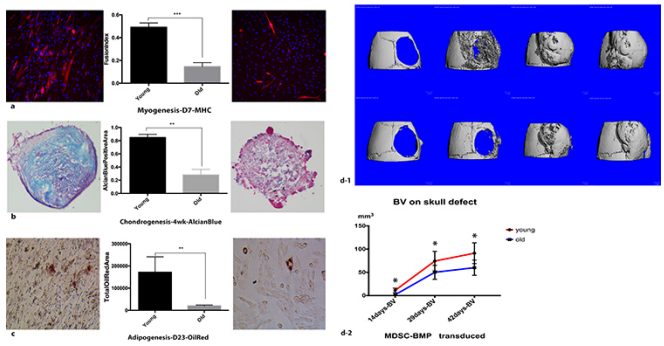

Figure1. a. Myogenesis Day7 muscle heavy chain immunostaining, quantified by fusion index under 20X. b. Chondrogenesis Day28 alcian blue staining, quantified by percentage of alcian blue positive area. c. Adipogenesis Day23 oil red staining, quantified by total area of oil red under 20X. a~c. Each shows sample pictures on both sides. d. In vivo bone regeneration using young and old BMP2/GFP transduced MDSCs in critical sized calvarial bone defect model. Bone regeneration were performed using microCT biweekly for 6 weeks.

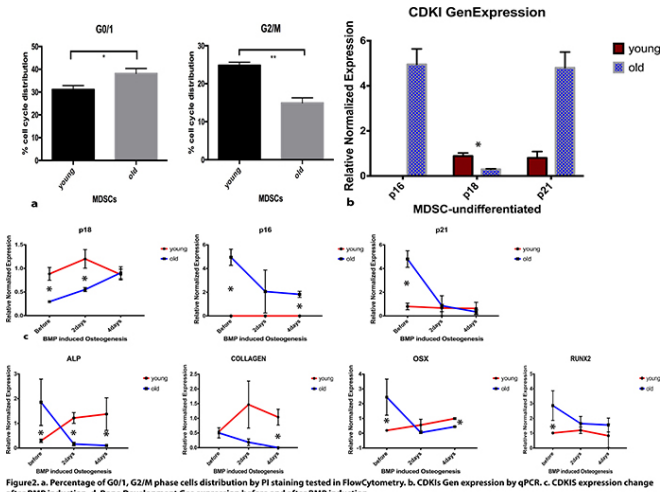

Figure2. a. Percentage of G0/1, G2/M phase cells distribution by PI staining tested in FlowCytometry. b. CDKIs Gen expression by qPCR. c. CDKIS expression change after BMP induction. d. Bone Development Ges expression before and after BMP induction

347. Transcriptional Profiling Helps to Uncover Molecular Mechanisms Implicated in Human Alveolar Mucosa Derived Cells Differentiation into Myoblasts

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Multipotent mesenchymal stromal cells (MMSCs) derived from various intraoral sources attracts attention of an increasing number of researchers due to availability and some features making them different from bone marrow or adipose tissue derived MMSCs. Myogenic potential of gingiva derived MMSCs was demonstrated for the first time in our recent experiments both at early and late passages. Identified subpopulation of cells was fully characterized by us using immunocytochemical staining, ELISA. It was shown that gingiva derived cells mathed generally accepted MMSCs criteria. Comparative analysis of gingival MMSCs and MMSCs obtained from another sources (including intraoral sources) testified the advantages of these cells. Further experiments showed that anatomical peculiarities play important role. Only MMSCs derived from alveolar (AMC) but not attached (AGC) part of gingiva are able to differentiate in myogenic direction. In order to reveal which molecular features distinguish between AMC and AGC we performed transcriptional profiling of human skin fibroblasts (taken as the norms), AGC, AMC (both primary and differentiated in myogenic direction), skeletal muscle cells (SMC) (both primary and differentiated in myogenic direction). When comparing AGC and undifferentiated AMC we identified 38 differentially expressed genes and 2 differentially regulated pathways. The differential genes did not form any specific functional Gene Ontology clusters. The most statistically significant genes were *NGT1*, *FERMT2*, *DYNLL1*, *GPLD1*, *TRAF5.* At the molecular pathway activation level, we found a differentially regulated branch of Wnt signalling pathway, namely, "WNT Pathway Cell Survival" and a branch of Chemokine signalling pathway, "Chemokine Pathway Gene Expression and Apoptosis via ELK1". The last one was also identified when comparing intact and differentiated AMC. Another two comparisons showed that this pathway is specific for AMC, but not SMC differentiation. Indeed, the same pathway was strongly differential among the differentiated AMC and SMC, and also showed differential scoring in the triple comparison (AGC, AMC, SMC). These findings evidence that the above pathway may have a strong implication in AMC differentiation. For the chemokine pathway branch, the most strongly differentially regulated node was the Focal adhesion kinase (FAK), represented by the products of the two genes- *PTK2* and *PTK2B.* The second most strongly differential gene between the AMC and AGC was *FERMT2.* We propose that the FAK-FERMT2 interplay may be one of the drivers of gingival MMSCs pluripotency.

The study was funded by Russian Science Foundation (project # 14-25-00166).

348. A Comparative Study of Four Bone Morphogenetic Proteins on the Ability to Promote Osteogenic Potential of Human Muscle-Derived Stem Cells

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INTRODUCTION: It has previously been shown that human muscle-derived stem cells (hMDSCs) are capable of multipotent differentiation [1]. Similar to mesenchymal stem cells, hMDSCs require bone morphogenetic proteins (BMPs) to initiate osteogenesis [2, 3]. The osteogenic induction of 14 bone morphogenetic proteins have been studied for murine myoblasts *in vitro* and *in vivo* and showed different osteo-inductive capacities [4]. While the effects of BMP2 and BMP4 on hMDSC osteogenesis have been investigated *in vitro* and *in vivo*, other BMPs have not been studied extensively [1]. This study examined the osteogenic inductive potential of four different BMPs using hMDSCs. **METHODS:** *Cell culture:* hMDSCs from a 31yo female donor were expanded in proliferation medium. *In vitro* osteogenic differentiation was performed as previously described [1]. *MicroCT scanning:* Mineralized pellet volume and density were quantified with micro CT 3D quantification software. *Histology:* Mineralization was detected using von Kossa staining. Osteocalcin IHC was performed to validate osteogenic differentiation. **RESULTS:** MicroCT analysis indicated that all BMPs were capable of inducing hMDSCs to undergo mineralization (Fig. 1A). Quantification of pellet volume indicated significantly more mineralization in BMP treatment groups compared to the no-BMP group ($P<0.001$). In addition, BMP9 induced significantly higher mineralization volume than BMP6. BMP6 appears to be less effective than any other BMP treatment group (Fig. 1B). No statistical difference in pellet density was observed between any BMP treatment groups, with the exception of the BMP9 group, which has a higher density than the BMP6 group (Fig. 1C, $p<0.05$). Von Kossa staining showed mineralization in all BMP groups (Fig. 2A). Osteocalcin IHC showed osteogenic differentiation in all the BMP treatment groups (Fig. 2B). **DISCUSSION AND CONCLUSION:** Our results indicate that all tested BMPs induced osteogenesis in hMDSCs. We found that BMP6 is the least efficient. BMP9 treatment resulted in significantly higher pellet volume than BMP6 treatment, and increased pellet density compared to BMP2 treatment. Although BMP2 and BMP7 have been in clinical trials for years, our study revealed that BMP9 may represent a potential alternative for enhancing hMDSC-mediated bone regeneration. Further study is needed to test the mechanism and *in vivo* bone regeneration mediated by these BMPs.

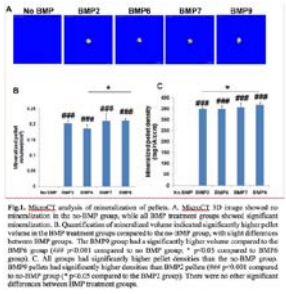

Fig.1. MicroCT analysis of mineralization of pellets. A. MicroCT 3D image showed no mineralization in the no-BMP group, while all BMP treatment groups showed significant mineralization. B. Quantification of mineralized volume indicated significantly higher pellet volume in the BMP treatment groups compared to the no-BMP group, with slight differences between BMP groups. The BMP9 group had a significantly higher volume compared to the BMP6 group (### p<0.001 compared to no BMP group; * p<0.05 compared to BMP6 group). C. All groups had significantly higher pellet densities than the no-BMP group. BMP9 pellets had significantly higher densities than BMP2 pellets (### p<0.001 compared to no-BMP group; * p<0.05 compared to the BMP2 group). There were no other significant differences between BMP treatment groups.

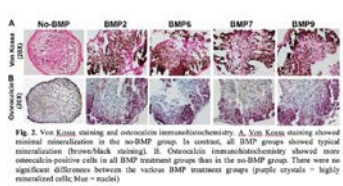

Fig. 2. Von Kossa staining and osteocalcin immunohistochemistry. A. Von Kossa staining showed minimal mineralization in the no-BMP group. In contrast, all BMP groups showed typical mineralization (brown/black staining). B. Osteocalcin immunohistochemistry showed more osteocalcin-positive cells in all BMP treatment groups than in the no-BMP group. There were no significant differences between the various BMP treatment groups (purple crystals = highly mineralized cells; blue = nuclei)

REFERENCES: [1] Gao X et al, 2013. [2] Gao X et al, 2014. [3] Wosczyna MN et al, 2012. [4] Kang Q et al, 2004.

349. Transplantation of HGF Modified Dental Pulp Stem Cell Prevents Bone Loss in the Early Phase of Ovariectomy-Induced Osteoporosis

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Transplantation of HGF modified Dental Pulp Stem Cell Prevents Bone Loss in the Early Phase of Ovariectomy-induced Osteoporosis Fanxuan Kong, Xuefeng Shi, Fengjun Xiao, Yuefeng Yang, Xiaoyan Zhang, Li-Sheng Wang, Chu-Tse Wu, Hua Wang Beijing Institute of Radiation Medicine, 27 Taiping Road,Beijing 100850, PR China Contact: Hua Wang, Ph.D., Department of Experimental Hematology, Beijing Institute of Radiation Medicine, 27 Taiping Road, Beijing 100850, PR China. Tel: +86-10-66932041 E-mail:wanghua@bmi.ac.cn; 18511712135@163.com Investigation based on mesenchymal stem cells (MSCs) for osteoporosis has obtained intensive attention recently. MSCs can be isolated from various tissues, such as bone marrow, adipose, umbilical cord, placenta, dental pulp, etc. Comparing to umbilical cord derived MSC (UC-MSC), bone-marrow derived MSC (BM-MSC) and adipocyte derived MSC (AD-MSC), dental pulp derived MSCs (DPSCs) and hepatocyte growth factor (HGF)-modified DPSCs (DPSCs-HGF) highly expressed osteogenic related genes, indicating stronger osteogenic differentiation capacity. Thus DPSCs have more benefits in treating osteoporosis. The purpose of this study is to investigate the roles of HGF and DPSCs in bone regeneration in ovariectomy (OVX)-induced bone loss mouse model. In this study, we transferred an adenovirus that carried HGF gene, luciferase gene or not carrying exogenous genes into human DPSCs. These cells were then transplanted into OVX-induced osteoporosis model for distribution observe or bone regeneration. Forty-seven mice received ovariectomy operation and twelve received a sham operation. After 4 and 6 weeks, μCT (micro-CT), qPCR (Quantitative RT-PCR) and ELISA were performed to compare the protective effect on osteoporosis mice treated with cell implantation. Observed by bioluminogenic imaging, we found DPSCs could survive over one month in vivo. The DPSCs mainly engrafted to lung in early stage and liver in late stage of administration and scarcely to the bone. There are more cells in vivo when DPSCs administrated early after OVX than late. DPSCs or DPSCs-HGF were transplanted and μCT analysis indicated that OVX induced significant bone loss in the trabecular bone of the distal femur metaphysis, and DPSCs-HGF transplantation could relieve the bone loss better. We also examined the serum markers related to bone remodeling, and discovered the expression of protein which activates osteoclasts increased in the OVX group. The expression of genes related to bone formation in bone marrow MSC were higher in DPSCs-Null and DPSCs-HGF group than in sham group. Our data suggested that systemic infusion of hDPSCs-HGF is a potential therapeutic approach for OVX-induced bone loss, which might be mediated by endocrine mechanism.

350. Genetically Modified Human Adipose-Derived Mesenchymal Stromal Cells (MSCs) Promote White Fat Conversion to Brown-Like Fat and Improve Glycemic Control in Diet Induced Obese Mice

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Background: Mesenchymal stromal cells (MSCs) are multipotent cells that home into inflammation sites. Upregulation of specific antioxidants in MSCs can prevent intracellular ROS accumulation, mitochondrial dysfunction and cellular inflammation in a hyperglycemic condition. **Hypothesis:** Antioxidant upregulation using gene overexpression in MSCs improves glucose homeostasis in high-fat diet induced obese (DIO) hyperglycemic mouse models. **Methods:** We used GFP-containing adenoviral constructs to upregulate intracellular (SOD1, SOD2, Catalase) and extracellular (SOD3) antioxidants in MSCs. Modified MSCs were delivered intraperitoneally into 60% and 45% fat fed DIO mice. **Results:** SOD2 and Catalase upregulated MSCs demonstrated the most improvement in glycemic control (glucose tolerance test) at week 4 in comparison to other antioxidants and Null-MSC (control). Catalase-MSC delivery not only improved glucose tolerance but also improved insulin tolerance in 60% fat fed DIO mice. Interestingly, RT-PCR of pericardial fat showed significant increases in mRNA expression of UCP1 (25-100,000-fold) and PRDM-16 (2-10-fold) among the 60% and 45% fat fed DIO mice that received antioxidants upregulated MSCs (Null-MSCs used as control). For omental fat, an increase in mRNA expression of UCP1 was also observed in 60% fat fed DIO mice (1,000-fold for SOD1 and SOD2; 3,000 and 6,000-fold for catalase and SOD3, respectively), while for 45% fat fed DIO mice only those receiving SOD1- and SOD2-MSCs presented UCP1 mRNA upregulation (1,000 and 11,000-fold respectively). Fat histology, particularly omental fat, showed less hyperplastic fat with SOD2- and Catalase-MSCs. UCP1 staining of omental fat was also positive with SOD2-MSCs. Plasma levels of both IL-6 and TNFa levels (by ELISA) were reduced with SOD2-MSCs in the 45% fat diet group. Additionally, TNFa was also reduced with SOD2-, SOD3-, and Catalase-MSCs in the 60% fat diet group. **Conclusion:** We conclude that delivery of antioxidant upregulated MSCs to the inflamed white fat depots in a DIO-dependent diabetes model appears to upregulate UCP1 and PRDM-16 while reducing systemic inflammatory markers. These findings may explain improvements noted in glucose and insulin tolerance. Therefore, delivering modified MSCs may be a novel yet safe therapeutic tool to improve glucose homeostasis which can be used to treat diabetes.

351. Human Adipose Derived Stem Cells Reduce Fibrosis in an Experimental Model of Chronic Kidney Damage Induced by Adenine

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Background and aims: Chronic kidney disease (CKD) exhibits fibrosis since early stages. Actually, there is no efficient treatment against renal fibrosis or renal failure, which is one of the main fifteen causes of worldwide death. In some experimental models of acute kidney damage, transplantation of autologous adipose tissue-derived stem cells (ADSCs) had demonstrated improving kidney function and reduction in fibrosis. In this study, we evaluated the antifibrotic effect of human adipose tissue-derived stem cells (hADSC) cell therapy in a CDK experimental model. **Methods:** Kidney damage was induced by daily orogastric administration of adenine (100mg/kg) to male Wistar rats during 28 days. hADSCs were isolated, expanded and characterized using flow cytometry and adipocyte and osteocyte differentiation before transplantation. hADSC administration was performed in tail vein at a dose of 2 $x10^6$ cells/animal. Animals were sacrificed 7 days post-treatment and blood, urine and renal tissue were collected. Percentage of fibrotic tissue, renal mRNA levels of Col-αI, TGF-β1, CTGF, α-SMA, IL-6, IL-10 and TNF-α , as well as, serum and urine levels of urea, creatinine and total protein were analyzed. **Results**: Treatment with hADSCs managed to restore renal levels of functional markers like creatinine, BUN and total protein ($p<0.01$). hADSCs transplantation reduced kidney fibrosis ($p<0.001$) and Collagen I deposition ($p<0.0001$) as observed in Masson and Sirius red staining. hADSCs administration diminished mRNA kidney levels of profibrogenic genes such as Collagen α1, α-SMA, TGF-β1 and CTGF ($p<0.05$). **Conclusions:** These results showed that cell therapy using hADSCs in a chronic model of renal fibrosis improves renal function and reduces fibrosis in a great proportion. Leading to the consideration of the use of hADSCs in pathologies involving renal fibrosis; thus may have clinical applications.

352. Cytoprotective Role of Oprd1 via Attenuation of UPR in Human MSCs Subjected to Oxidative Stress

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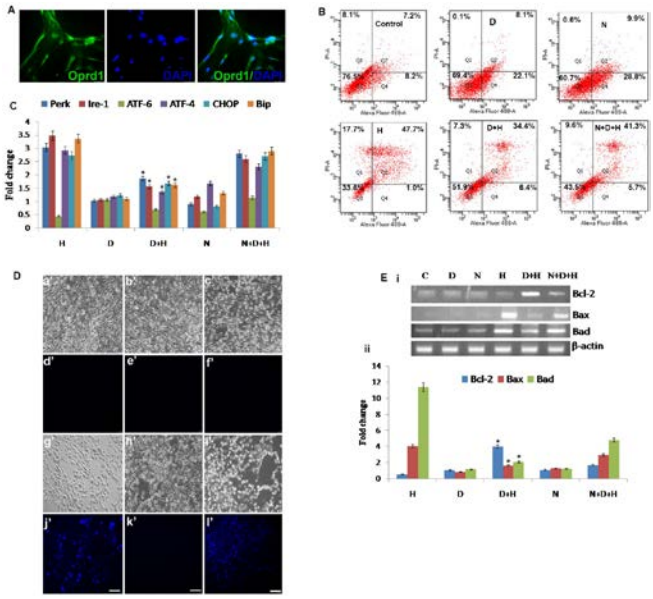
Human mesenchymal stem cells (hMSCs) have shown promise in repairing injured or ischemic tissues. But their successful transplantation is hindered by the paucity in their viability, which in turn compromises the efficacy of stem cell therapy. An increased level of reactive oxygen species (ROS) in damaged sites prove detrimental to the survival of donor MSCs. In the present study, the effect of delta-opioid receptor (Oprd-1) activation on hMSC (derived from umbilical cord blood) survival under oxidative stress (H_2O_2) was evaluated. Oxidative stress is known to trigger pathological conditions of the unfolded protein response (UPR) leading to Endoplasmic Reticulum (ER) stress. In the current study, upon Oprd-1 activation by D-Alanine 2, Leucine 5 Enkephaline (DADLE) in hMSCs exposed to oxidative stress, there was a significant downregulation (~2folds) in key UPR gene expression levels as determined by quantitative (q) PCR as well as Thioflavin-T protein aggregation assay (Fig 1A, C & D). Concomitantly, the oxidative stress mediated cell-death was ameliorated and the percentage of cell viability was enhanced following Oprd1 activation compared to the control (~52 % vs 34%) (Fig 1B). The increased intracellular ROS production upon H_2O_2 treatment as determined by Chloromethyl derivative of 2', 7' -dichlorofluorescindiacetate (CM-H_2DCFDA) was also significantly suppressed following Oprd1 activation. In addition there was ~8 fold increase in the expression levels of the anti-apoptotic marker gene, Bcl-2 as well as a significant repression in the expression levels of pro-apoptotic genes Bax and Bad (~3 and 5 folds respectively) upon Oprd1 activation compared to H_2O_2 alone treated control as determined by qPCR (Fig 1E). All the above mentioned pro-survival effects were significantly neutralized by naltrindole (highly specific Oprd1 antagonist) indicating that DADLE may act through the Oprd1 to exert its cytoprotective effects. These results suggest the possible role of Oprd1 in the improvement of hMSC viability that might possibly help sustain successful cell transplants into the injured tissues and greatly absolve the inefficacy of stem cell transplantation. **Fig 1: (A)** Expression of Oprd1 in human umbilical cord blood derived MSCs (hUC-MSCs) as determined by immunofluorescence, green-Alexa Fluor 488, Blue-DAPI. **(B)** Cell viability under different treatments measured by Annexin V/PI using flow cytometry. **(C)** Relative fold change (normalized to 1 for the untreated control) of key UPR genes under the different treatment conditions, *$p<0.05$ vs control (only H treated). **(D)** Thioflavin-T protein aggregates under the different treatment conditions. Phase contrast images of (a') untreated control, (b') only D, (c') only N, (g') only H, (h') D+H and (i') N+D+H treated cells; (d'-f') corresponding fluorescent images of a', b' and c' respectively; (j'-l') corresponding fluorescent images of d', e' and f' respectively. **(E)** i- semiquantitative PCR gel image of the different genes, ii- qPCR data for the different genes under the various treatment conditions. *$p<0.05$ vs control (only H treated). C= untreated control, D= DADLE, N- Naltrindole, H- H_2O_2



353. Anti-Oxidative Activity and Protective Role of Human Placental Mesenchymal Stem Cell-Derived Extracellular Visicles in Murine Acute Lung Injury Model

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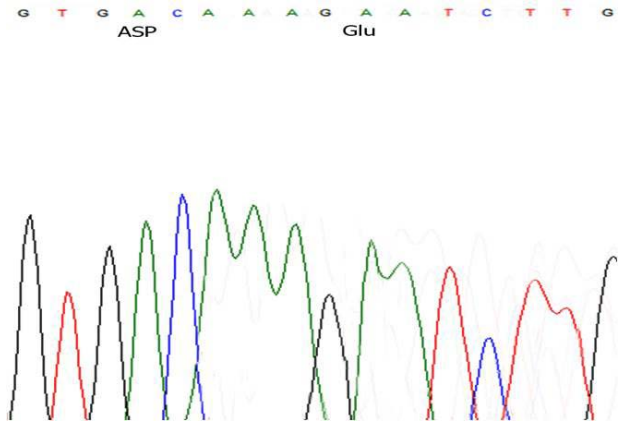
Acute Lung injury (ALI) remains a devastating syndrome caused by overwhelming inflammatory responses with a mortality rate over 50% in China. Recent studies demonstrated that mesenchymal stem cells (MSCs) exerted their biological functions of immunosuppression injury repair, mainly through the release of extracellular vesicles (MSC-EVs) that containing factors and genetic materials with various biological activities, including mRNAs and microRNAs (miRNAs). In order to investigate the potential and mechanism of human fetal placenta mesenchymal stem cells (fPMSCs) for ALI treatment, the anti-oxidative capacity and protective role the extracellular vesicles (fPMSC-EVs) collected from fPMSCs under a condition of serum free culture were analyzed *in vitro* and *in vivo* using an ALI murine model. By using vitamin C (Vc) as a control of anti-oxidant agent, the total antioxidant capacity (T-AOC), scavenging capacity of free radical and antioxidant enzymatic activities of fPMSC-EVs were evaluated. In comparison with Vc, fPMSC-EVs exhibited obvious antioxidant capacities at different extents between passages of cell cultures. The total antioxidant capacity (T-AOC) of the culture supernatant was comparable to 40-80 mol/L of Vc. In addition, both fPMSC culture supernatants and fPMSC-EVs displayed capacities to scavenge radicals, including 2,2-diphenyl-1-picrylhydrazyl (DPPH·), hydroxyl radical (·OH), superoxide anion radical (O^{2-}), along with an increased activity of antioxidant enzymes, including superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX). Even more, both culture supernatants and EVs of fPMSCs could alleviate H2O2-induced lung epithelial cell injury in vitro. Encouragingly, an intravenous injection of fPMSC-EVs showed a reduced lung damage and inflammatory responses in LPS-induced ALI in mice, as determined by morphological analysis and cytokine productions, suggesting that fPMSC-EVs may have a protective role in mice from LPS challenge. These results indicate that fPMSC-EVs are potential cell-free therapeutic agents that warrant for further investigation.

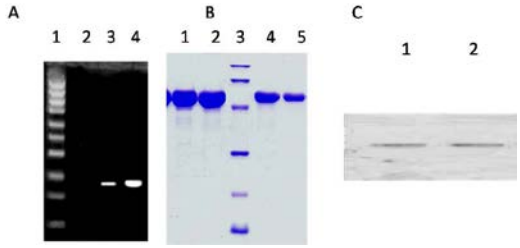
354. Protein Engineering

Naghmeh Bahrami[1], Mohamad Bayat[1], Abdolreza Mohamadnia[2], Sahar Shojaee[3], Naeim Bahrami[4]

[1]Craniomaxillofacial Research Center, Tehran University of Medical Sciences, Tehran, Iran, Islamic Republic of, [2]Virology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran, Islamic Republic of, [3]Cell Therapy Center, Gandhi Hospital, Tehran, Iran, Islamic Republic of, [4]University of California, San Diego, Department of Psychiatry, La Jolla, CA

Abstract: The aim of the present study was to assess the recombinant bone morphogenetic protein 2 (RHBMP-2) with higher substantively and solubility for using in calcium phosphate scaffolds for better releasing in differentiation of mesenchymal stem cells to osteoblast cells. By using bioinformatics tools, 2 mutations (p. L10D and p. S12E) were chosen and applied in BMP2 CDS sequence to increase interaction with calcium derived composite. The new recombinant mutated sequence ($BMP2^{mut}$) was synthesized and then sub cloned to expression vector pBV220. Experimental data regarded functional protein expression in E.Coli. Since no modification was made in the active sites of proteins namely β-sheets and α-helixes, not only no changing occurred in the specific activity of the enzyme in comparison to its commercial counterpart but also mesenchymal osteogenesis was done more efficient on biphasic CaP scaffold. As we hypothesized, use of negatively charged amino acids such as aspartate and glutamate in protein loops increased the interactions of BMP2-Ca^{2+} and resulted in its slower and more sustained released from CaP scaffolds compare to commercial RHBMP2. Our data suggested that new $BMP2^{mut}$ have greater osteoinductive capacity than RHBMP2 in the same time and dose than RHBMP2.





355. Manufacturing Solutions for Autologous Cell Therapies

Samantha Luther, Sandhya Punreddy, Tristan Lawson, Anjali Verma, Susan Rigby, Aletta Schnitzler, Martha Rook, Julie Murrell

MilliporeSigma, Bedford, MA

Cell expansion solutions that are robust and compatible with limited downstream processing steps will be necessary for the manufacturing of regenerative medicine therapies progressing through clinical trials and into commercialization. In this study we describe implementation of single-use bioreactors and high-quality media for expansion of cell therapies with a focus on autologous T cells. We investigated the impact of different culture platforms and media formulations on expansion of CD4+ T cells. Expansion of T cells in stirred-tank bioreactors ranged between 70 to 110-fold. A wide range of performance was observed between media formulations, and positive performance in planar culture was not necessarily predictive of that in suspension cultures. These results demonstrate that industrialization paradigms can be applied to autologous therapies. Single-use devices and high quality animal origin-free reagents support the future implementation of manufacturing solutions that will be required following clinical success.

356. EpiX™: A Primary Epithelial Cell Bioproduction Technology Providing Genetic Stability, Cost Efficiency, and Enablement of *Ex Vivo* Genetic Engineering

Chengkang Zhang, Ruipeng Wang, Anura Srivastava, Brian Pollok

Propagenix Inc., Rockville, MD

Generating sufficient cellular biomass remains a central and elusive challenge in cell therapy and regenerative medicine. Propagenix has developed a technology platform called EpiX™ which provides for unprecedented expansion of many types of human primary epithelial progenitor cells. Notable attributes of the technology are:

- A chemically-defined formulation lacking feeder cells, serum, or xenobiotic agents

- Preservation of genetic integrity after extensive cell expansion, as assessed by karyotype and whole-genome sequencing

- No tumorigenic properties and no selection of tumor driver gene mutation after extensive cell expansion

- Efficient single cell cloning and a proliferative runway enabling sophisticated genetic engineering to enhance cell function

- And very importantly, ready differentiation of EpiX™-expanded cells to their committed cell differentiation fate upon removal from growth conditions.

Here we present data demonstrating the cell expansion properties of EpiX™ technology for primary human keratinocytes, airway cells, and additional epithelial cell types. Whole genome sequencing of primary keratinocytes evidenced no heightened mutation rate or cancer driver gene mutation after extensive expansion in EpiX™ media. The absence of *in vivo* tumorigenicity of EpiX™-expanded primary cells similarly shows a preferred safety profile for this cell expansion technology. Finally, we show how primary human epithelial cells can be engineered *ex vivo* to be protected from destruction by a trained allogeneic T-cell response. This "immune cloaking" approach has broad relevance for potentially preventing immune rejection when using allogeneic epithelial cells for certain tissue grafts and may provide a path to creating "off the shelf" allogeneic cell therapies.

357. Serum Starvation Increase Levels of HSPs and Cancer Stem Cell Markers in Esophagus Cancer Cell Line

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Cancer stem cell hypothesis describes a subset of cells responsible for tumor initiation and maintenance, their rises from normal stem cells or other cells is remain to be elucidated. They contribute to major hallmarks of cancer and earned much attention since they make this disease harder to cure. The correlation between cancer stem cells and stresses it's encounters in tumor microenvironment such as hypoxia, serum starvation and hyperthermia is unclear. Such stresses attribute to cancer cell death, plasticity and epithelial mesenchymal transition. Its well established now that heat shock proteins such as HSP70 and HSp90 play a substantial role in cancer cell resistant to stress thereby elevating tumors invasiveness, antiapoptosis, and drug resistant. In this research we asked if the starvation conditions which cancer cell encounter in tumor microenvironment could be the driving force attribute to cancer stem cell subpopulation, and if this so, are they related to HSP70 and HSP90β levels. Esophagus cancer cell line SKGT4 was used as model. Cultured cells were divided into two types of conditions; first subcultures were under normal growth conditions in RPMI-1640 with L-glutamine, glucose, and 10% FCS. The second subcultures were under starvation conditions in RPMI-1640 media without L-glutamine, glucose, and FCS. Both incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 and 48hr and extended incubation time 72 and 96hr. MTT assay was used to determined reduction in cell viability. After the end of each incubation time for both incubation conditions, cells were harvested to determine levels of mRNA of HSP70, HSP90β, CD44, ALDH, and ABCG2 using one-step quantitative reverse transcriptase PCR (qRT-PCR). Results indicated a loss of cells viability during the extended time of incubation. There was continuous decline of cell viability in the presence of 10% FCS the reduction of viability was 24.95% and 54.77% at 72hr and 96hr respectively. Cell viability reduction increased to 49.33% and 62.41% at 72hr and 96hr respectively in serum starvation condition (fig. 1). In regard to genes

under study, a significant correlation between serum starvation for extended time of incubation 72hr and 96hr and elevated levels of mRNA. For stress proteins, HSP70 found to be significantly elevated at extended time of incubation in both cultivation conditions but it was higher in starvation conditions. The same was true for HSP90β, the exception was in the non-starvation conditions after 72hr, levels were not significant during 24, 48 hr of incubation time. Obviously HSPs responded for both starvation and extended incubation time in this cell line (fig. 2). Cancer stem cell markers CD44, ABCG2, ALDH genes witnessed elevation in its mRNA in both starvation conditions and prolonged incubation time (fig. 2) the three markers significantly increased ($p \leq 0.01$). Totally these data suggest that starvation taking place in tumor microenvironment as a result of shortage in resources for growth factors (serum) and glucose induces mechanisms in cancer cells may render them new capabilities.

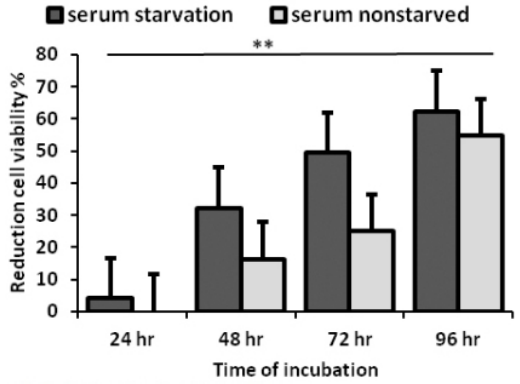

Fig.1: Reduction in cell viability

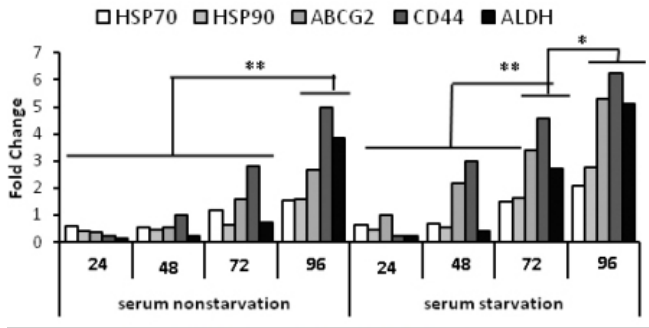

Fig.2: Fold change in levels of mRNA

Gene Targeting and Gene Correction II

358. Maximizing Translation of Cas9 mRNA Therapeutics by Sequence Engineering and Chemical Modification

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Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 is an exciting new system for facile gene inactivation or genome engineering. Both types of applications require delivery of Cas9 protein and a RNA guide to the nucleus of cells. Often for clinical applications, a chemically synthesized guide RNA is co-transfected with a Cas9 messenger RNA (mRNA) into cells or organisms. For maximal expression in cells or target organs, transfected mRNAs must avoid detection by pattern recognition receptors (PRRs) that evolved to sense improperly capped RNAs and double stranded RNA. PRR activation leads to cytokine production, translational arrest and cell toxicity or death. Mammalian mRNAs are modified post-transcriptionally to contain modified nucleotides (e.g. pseudouridine and 5-methylcytidine). These modifications can reduce activation of PRRs and allow maximal translation of the transfected mRNA. During RNA capping, Cap0 (m7GpppN) is formed as an intermediate. Methylation of the 2' position of the first nucleotide forms Cap1, which is found in ~50% of endogenous mRNAs. mRNAs generated with commercially available cap analogs (ARCA) contain Cap 0 structures that can be immunogenic. Recombinant enzymes used to generate Cap1 mRNA are expensive, do not always go to completion and the RNA must be purified prior to capping. We recently developed a novel co-transcriptional capping method called CleanCap that yields Cap1 with high efficiency and lower costs in a "one pot" reaction. First generation Cas9 mRNAs were modified with pseudouridine or 5-methylcytidine/pseudouridine and had Cap0 structures. We generated improved second generation Cap 1 mRNAs through a combination of sequence engineering and screening of chemical modifications. In all, 18 combinations were tested with and without HPLC purification. These RNAs were assayed for double stranded RNA contamination, translation efficiency in wheat germ extracts, protein expression and interferon production in THP-1 cells and indel formation in primary CD34+ cells. Interestingly, wild-type, pseudouridine and 5-methyluridine containing mRNAs induced an interferon response in differentiated THP-1 cells. Through these studies we were able to dramatically increase indel formation in CD34+ cells relative to first generation mRNAs. Indel formation as high as ~85% was observed. By comparison, recombinant Cas9 protein complexed to guides gave ~65% indel formation. Interestingly, some modified RNAs did not require HPLC purification for maximal activity.

359. CRISPR-Based Gene Correction to Treat IPEX Syndrome

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Primary immunodeficiencies (PIDs) comprise a group of diseases in which the immune system does not develop properly, leading to immune manifestations such as the inability to fight infections and the development of autoimmunity. The prototypical PID with autoimmunity is IPEX syndrome, which is a severe, and often fatal, pediatric disease caused by mutations in the *FOXP3* gene. Currently, the only effective treatment for IPEX is allogeneic hematopoietic stem and progenitor cell (HSPC) transplantation from a related or unrelated donor. However, many patients either do not have a suitable donor or have severe transplant-related complications. To address the current limitations, we propose autologous HSPC transplantation for IPEX, whereby patient HSPCs are gene corrected ex-vivo and reinfused into the patients. Due to the widespread distribution of patient-specific mutations throughout the *FOXP3* gene, here we designed a gene repair strategy that uses homology directed repair to insert, or knock-in, the coding sequence of the *FOXP3* gene at the start codon of the mutated gene. This strategy can permit the regulated expression of the wild-type, functional FOXP3 protein in patient cells independent of the location of the downstream mutation. To accomplish site-specific gene knock-in, we used a combination of Cas9, a chemically modified sgRNA targeting *FOXP3*, and an AAV6 packaged homologous donor DNA template, and showed that the system effectively targets *FOXP3* in primary human HSPCs. To test for functional correction of *FOXP3*, we used the CRISPR system to target the major cell type that relies on *FOXP3* expression, thymic-derived T regulatory cells (Tregs). Gene editing of normal donor and IPEX Tregs and T conventional cell allowed us to test for regulated gene expression and for establishment of normal Treg suppressor function and T cell proliferation upon activation. Additionally, we demonstrated that gene edited HSPCs can be transplanted into NSG mice for long-term multilineage reconstitution. The results from the study support the use of CRISPR/Cas9 as a therapeutic approach to treat IPEX syndrome patients. This gene editing approach may also be applied to treat other pediatric monogenic blood and immune disorders.

360. CRISPR/Cas9-Mediated Correction of the Human Disease Herediatary 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, a gene correction frequency of 11 to 25% of hepatocytes was achieved. The efficiency in adult mice was significantly lower, <1%. Nonetheless, expansion of gene-corrected hepatocytes protected the mice from developing the liver disease. These results indicate that CRISPR-Cas9-mediated genome editing is a promising approach for correction of human genetic diseases of the liver. Multiple small molecules that could potentially enhance the correction efficiency *in vivo* are currently under test in both neonatal and adult mice. Gene repair vectors capable of targeting the human albumin locus have also been designed and are being tested in chimeric mice repopulated with human hepatocytes.

361. High Frequency of Sero-Positivity of IgG Antibodies Against Cas9 Protein in Humans

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The bacterially derived CRISPR/Cas9 system has proven to be a powerful tool for genome editing and its' *in-vivo* use has shown promise for treating human disease. The most commonly used Cas9 systems are derived from *Streptococcus pyogenes* (*S. pyogenes*) and *Staphylococcus aureus* (*S. aureus*), bacterial species that inhabit humans. Infections caused by these bacteria are common raising the possibility that IgG antibodies against Cas9 may be present within the human population. To look for pre-existing antibodies against S. aureus Cas9 (SaCas9) and S pyogenes Cas9 (SpCas9) in humans we used human cord blood serum to probe against purified SaCas9 and SpCas9. Probing against purified Cas9 with human serum from 22 donors at a dilution of 1:10 we found that 86% of donors stained for SaCas9 and 77% of donors stained for SpCas9. The specificity of the antibody staining was demonstrated by showing immunoreactivity to CD34+ cells that had been electroporated with SpCas9 and SaCas9 protein but not to CD34+ cells that had not been electroporated. Our results demonstrate that IgG antibodies against SpCas9 and SaCas9 are present within the population raising an important potential barrier to using either Cas9 homolog as part of an in vivo genome editing strategy.

362. DeepSanger: A Rapid, Cost-Effective Alternative to Next-Generation Sequencing for Indel Identification and Quantification

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Programmable genome editing tools such as TAL effector nucleases (TALENs), zinc finger nucleases (ZFNs) and CRISPR-Cas nucleases enable efficient introduction of precise DNA double-strand breaks (DSBs) and induction of genome editing events. However, the resulting mutagenic repair outcomes from non-homologous end-joining (NHEJ) can be quite heterogeneous at a target locus, due to the induction of variable-length insertion or deletion mutations (indels). Most existing assays for assessing indel mutations either provide limited information or require access to more costly next-generation sequencing technology. Enzymatic assays such as Surveyor and T7 Endonuclease I (T7E1) allow for relatively inexpensive semi-quantitative assessment of indel mutation frequency at a given target site, but perform poorly at high cleavage rates and don't provide information about the indel sizes or sequences. Other methods such as Tracking of Indels by DEcomposition (TIDE) and Indel Detection by Amplicon Analysis (IDAA) are also cost-effective and can provide information about the size of indels but also do not provide sequences of the mutations. Targeted amplicon sequencing using next-generation sequencing methods is the current state-of-the-art method for quantifying indel frequencies and yields actual mutation sequences; however, this method is laborious, requires access to more expensive next-generation sequencing platforms, and necessitates informatics analysis of the resulting data. To address the limitations of existing methods, we have developed a robust, rapid, and relatively inexpensive method for indel mutation quantification and sequence determination that we call **DeepSanger**. This new method can be easily practiced using standard PCR and Sanger sequencing with data processing performed with a publicly available, user-friendly software program that we developed specifically for this assay. In direct comparisons, DeepSanger yields indel mutation sequences and frequencies that are highly correlated with the results of targeted amplicon methods performed with next-generation sequencing. In addition, DeepSanger can reliably detect mutations with frequencies of 1% or lower, highlighting the capability of the method to identify even rare indel events. The capability of DeepSanger to provide the specific sequences of indel mutations stands in contrast to TIDE and IDAA, neither of which can provide this additional important information. DeepSanger can also be used with different CRISPR-Cas orthologues as well as CRISPR-Cpf1 nucleases, enabling extension of the method for a wide range of applications; this flexibility contrasts with TIDE, which can only be used to assess indels created with the *Streptococcus pyogenes* Cas9 (SpCas9). We will also describe publicly available web-based software tools and protocols that can be used to practice the DeepSanger method. In summary, DeepSanger provides a simple, inexpensive, and robust solution for identifying and quantifying indel mutations that closes the gap between more limited screening tools such as Surveyor/T7EI assays, TIDE, or IDAA and more laborious and costly next-generation sequencing-based approaches.

363. Regulating the Expression of Therapeutic Transgene by Controlled Intake of Dietary Essential Amino Acids

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Widespread application of gene therapy will depend on the development of simple methods to regulate the expression of therapeutic genes. Here we harness an endogenous signaling pathway to regulate therapeutic gene expression through diet (Chaveroux et al 2016). The GCN2/eIF2a/ATF4 signaling pathway is specifically activated by deficiencies in any essential amino acid (EAA); EAA deficiency leads to rapid expression of genes regulated by ATF4-binding *cis* elements (called Amino Acid Response Elements - AARE). We found that therapeutic genes under the control of optimized AAREs had low basal expression and high-induced expression. Briefly, after consumption of a diet deficient in one EAA, the blood concentration of the limiting EAA decreases rapidly and greatly, triggering the activation of the GCN2/eIF2a/ATF4 pathway and then the AARE-dependent transcription. For regulation of therapeutic transgenes over long time periods we performed pulses of amino- acid-deprived diet, swapping each of the lacking EAA.As a first proof of concept, we showed that the AARE- driven expression system can efficiently regulate the expression of a reporter gene (Luciferase) in liver, pancreas and brain. As a second proof of concept we applied our system to regulate the expression of *TNFSF10* (*TRAIL*) in the context of glioma therapy and found that intermittent activation of this gene by EEA-deficient meals retained its therapeutic efficacy while abrogating its toxic effects on normal tissue. Such a system, based on dietary specific amino acid starvation, does not require the expression of synthetic transcription factor or regulatory proteins nor the administration of pharmacological inducers. It is physiological, non-toxic and is amenable to clinical application. The characteristics and benefits of the "nutrition-based" regulatory system are: (1) precise control of the induction period, (2) very low basal expression level of the transgene expression and a high induced expression level, (3) control of transgene expression in several tissues, and (4) absence of toxicity. The temporal regulation of therapeutic gene expression has long been awaited to broaden the clinical utility of gene therapy. In this study, we showed how a regulatory system, initially discovered in the context of basic studies pertaining to the physiology of nutritional stresses, provides a simple and robust means to control the expression of exogenous transgenes. Our system may be particularly well suited for intermittent regulation of therapeutic transgenes over short or long time periods. It exhibits the desirable properties for translation from the laboratory to clinical practice. Reference: Chaveroux et al (2016) Nature Biotechnology Jul;34(7):746-51

364. Self-Inactivating Cas9: A Method for Reducing Exposure While Maintaining Efficacy in Virally-Delivered Cas9 Applications

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Genome editing technologies hold great promise for treating genetically defined disease at the genomic level. Recent work has shown that the smaller Cas9 ortholog from *Staphylococcus aureus* can be packaged with two gRNAs and all of the appropriate control machinery in a single, all-in-one AAV. Unlike traditional gene therapy, in which consistent expression of a transgene may be desirable, in a genome editing setting it may be preferable to express Cas9 in the cells of interest only until the target locus has been modified at one or both alleles; any further expression would be unnecessary. While AAV is generally considered to be non-integrating, it can persist for extended periods of time, providing sustained expression of its payload. Here we have further engineered our AAV vector system such that it contains self-inactivating, universally applicable, tunable modules. These modules parsimoniously include the already-targeted endogenous sequence, obviating the need for any additional gRNAs, and can be tuned based on position within the viral genome, choice of gRNA, or PAM sequence. Our constructs provide for robust Cas9 expression and target locus modification while simultaneously self-targeting the pool of AAV DNA at its source. Both *in vitro* and *in vivo* results show promise for this method and demonstrate the feasibility of attaining robust target modification with substantial reduction of exposure through time in a target tissue.

365. Fine and Predictable Tuning of TALEN Gene Editing Targeting for Improved T-Cell Adoptive Immunotherapies

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One of the major challenge of gene editing using designer nucleases, especially for clinical applications, is the possibility of off-target cleavage. Thus, our capacity to control and finely tune the targeting specificity of any genome engineering platform represents a key issue. TALEN have already proven to be extremely specific and compatible with ex-vivo engineering of primary T-cells for therapeutical applications, with no or very low (background) levels of off-site processing. So far, 4 DNA targeting modules (RVDs) are mainly implemented and used by most researchers (NI, HD, NN, and NG) but these naturally found RVDs only explore about 5% of the possible diversity repertoire. Recent studies took advantage of the extended repertoire offered by the DNA targeting characteristics of RVDs (position 12 and 13), to identify and characterize new non-conventional RVDs (ncRVDs) with novel intrinsic targeting specificity features. This strategy to control TALEN targeting is based on the exclusion capacities of these ncRVDs. Recent studies underlined that disruption of endogenous PD-1 using designer nucleases enhances the efficacy of gene-disrupted CAR T-cells therapy. Here we implemented the ncRVD approach to edit the genome of human primary T cells with TALEN presenting improved discrimination between the desired on-site and potential off-sites targets. In particular we designed a novel TALEN that demonstrated very high level of processing of its cognate PD1 target while abolishing processing of a low frequency target. We further demonstrated that the ncRVD technology could be applied to combine new (with ncRVD) and already designed (without ncRVD) TALEN for multiplex editing experiments. As we previously described a platform for the mass-production of "off the shelf" CART cells from unrelated third-party donor T cells by disrupting the TRAC gene we focused on designing a TALEN targeting the PD-1 locus and that can be used in combination with this previously reported TRAC TALEN. Altogether, these results showed that a very fine and predictable tuning of a TALEN targeting can be obtained by incorporating few ncRVDs, further extending the possibilities of this genome editing platform.

366. Development of a High-Throughput Sequencing Approach for Off-Target Assessment in Therapeutic Genome Editing Applications

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The adoption of CRISPR in the field of genome-editing is revolutionizing biomedical research, providing a powerful tool for precise genome modification. It has demonstrated increased benefits over previous iterations, such as TALENs and zinc finger nucleases (ZFNs), due to low cost, relative simplicity and higher efficiency at performing bi-allelic genome modifications.

Given the exponential use of CRISPR in research and therapeutic applications, there is a need to understand both intended and genome-wide outcomes of CRISPR experiments. Off-target editing events impact experimental outcomes by introducing double-stranded breaks in regions that may lead to genome instability, disruption of gene regulation, cell death or tumorigenesis.

We have developed a high-throughput next-generation sequencing approach for investigating off-target effects of CRISPR genome-editing experiments. This approach involves the design of Tru-Seq Custom Amplicon (TSCA) primers for multiplexed target amplification and deep sequencing of predicted off-target sites. In this study, the specificity of sgRNAs targeting the RAG2 gene were assessed in K562 myelogenous leukaemia cells and CD34+ human pluripotent stem cells. TSCA primers were designed targeting 125 predicted CRISPR editing sites. A total of 3000 amplicons were pooled for parallel deep sequencing on a single Mi-Seq run. FASTQ data was demultiplexed and predicted sites were analysed using a custom bioinformatics pipeline.

Results show negligible cross-hybridization across TSCA primer pairs indicating high specificity to the target loci. Over 80% of amplicons are seen to achieve uniform coverage, negating target region bias across the 125 predicted sites. CRISPR editing events were detected at the

predicted on-target site as well as several predicted off-target sites. Mutations in the population were detected to a sensitivity of 0.1% minor allele frequency.

These results indicate the advantage of the TSCA assay for high-throughput off-target assessment in therapeutic genome editing applications. This presents a novel approach for investigating genome-editing incidence rates and associated repair products. We suggest this approach as a scalable option for discovering, validating, and assessing the safety of genome-editing modifications compared to current assays.

367. Efficient Generation of CAR T Cells by Site-Specific Gene Addition into the TRAC Locus via Homology-Directed Repair

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Genetically engineered T cells represent a promising new approach to the treatment of cancer. Positive results in recent clinical trials exploiting T cells engineered to express chimeric antigen receptors (CARs) have highlighted the potential for these cell-based therapies in patients with B cell malignancies. To extend these successes to a broader range of tumor types may require additional T cell engineering beyond simple CAR addition, such as gene knockout and/or coupling deletion of a target gene with site-specific addition of a CAR transgene. To this end, we have developed a genome editing strategy for the simultaneous elimination of an endogenous target gene with site-specific addition of a CAR via homology-directed repair (HDR). To demonstrate the utility of this approach, we used a previously characterized megaTAL (an engineered nuclease created by the fusion of an engineered meganuclease and a transcription activator-like (TAL) -DNA binding domain) specific for the T cell receptor alpha constant region gene (TRAC). Delivery of this megaTAL to primary human T cells via mRNA electroporation results in efficient and specific disruption of the TRAC locus (Boissel et al, 2013). To achieve simultaneous target gene disruption with site-specific CAR transgene insertion, we designed an adeno-associated virus (AAV) vector for DNA template delivery encoding the CAR and flanked by regions of DNA homologous to the genome immediately surrounding the megaTAL cleavage site. Co-delivery of the megaTAL and AAV encoding a CD19-CAR with TRAC homology arms resulted in >50% CAR+TRAC- cells. *In vitro* assays of cytotoxicity and cytokine responses against CD19+ Nalm-6 cells confirmed that TRAC-targeted CD19-CAR T cells were indistinguishable to CD19-CAR T cells generated by lentiviral transduction. Interestingly, a similar level of CAR T cell function was observed even though TRAC-targeted CD19-CAR T cells expressed lower amounts of the CAR, as determined by flow cytometry. Similar CAR integration efficiency and functional efficacy was observed using a TRAC-targeting AAV vector containing a distinct B cell maturation antigen (BCMA)-specific CAR. These findings demonstrate megaTAL-mediated targeted gene addition as a feasible, efficient, and potentially safer approach for generation of gene-edited CAR T cell product. Moreover, the ability to combine the disruption of a target gene with the site-specific integration of the CAR eliminates the need for randomly integrating viral vectors while satisfying the potential need for more complex genome-edited T cell pro

368. Highly Efficient Genome Editing for Single Base Mutation in hiPSCs Using CRISPR/Cas9

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The target specific genome editing in induced pluripotent stem cells (iPSCs) holds promise in wide variety of fields. Single base mutations using single-stranded oligo DNA nucleotides (ssODNs) can be used for introducing and/or correcting disease-associate mutations to generate human disease model for drug discovery and elucidation of diseases. Precise mutations can be achieved by homology-directed repair (HDR), but the efficiency is usually very low, which hampers development toward clinical application. For successful genome editing in iPSCs, improving the editing efficiencies, increasing the single cell cloning efficiencies, and maintaining iPSCs in an undifferentiated state throughout the whole process are essential. In this study, to improve the knock-in efficiency with ssODNs using CRISPR/Cas9 system, we have established the simple evaluation systems for quick detection of accurate single base mutations by HDR and insertion/deletion mutations simultaneously using a flow cytometer in both 293T cells and human iPSCs (hiPSCs). Using these systems, we have investigated the optimal design of ssODNs such as the blocking mutation at various position, orientation, size, and length of homology arms. First using plasmids expressing Cas9 and sgRNA, we have demonstrated ssODN having a mutation at PAM showed the highest knock-in efficiencies, and there was the inverse correlation between the distance from PAM to the mutation site in ssODNs and the knock-in efficiencies. These results indicate re-cutting of the corrected gene is the major cause for very low efficiencies, and the block mutation in ssODNs to prevent re-cutting is essential for efficient HDR. We have also shown that ssODNs complementary to gRNA strand showed higher knock-in efficiencies, and longer homology arms improved the HDR efficiencies, but longer ssODNs lowered the efficiencies because of the cytotoxicity. The balance of the HDR efficiencies and the cytotoxicity of ssODNs is important for efficient genome-editing. The optimized ssODNs showed more than 10 times higher knock-in efficiencies compared to the ssODNs having no blocking mutation. Recently the delivery of Cas9 protein/gRNA ribonucleoprotein complexes (RNPs) is reported to show more efficient on-target cleavage and reduce the off-target cleavage compared to plasmid transfection. Furthermore, genome editing using RNPs can solve the issues such as random integration and insertion at on-/off-targets sites of plasmids into the genome, and severe cytotoxicity caused by introduction of nucleic acids. We have demonstrated RNPs could improve the genome editing efficiencies in both knock-out and knock-in, using our novel 293T and hiPSC models. The knock-in efficiency was the highest when using ssODNs with the mutation at PAM site or at 5' neighboring base of PAM as donor DNAs, similarly to the results using plasmids. However, the reduction of knock-in efficiency with ssODN having no blocking mutation were much smaller compared to plasmids, indicating RNPs could dramatically reduce the re-cutting of the edited sites. In addition to the improvement of genome-editing efficiencies, the optimal system for single cell cloning step to promote expansion of the clones and maintain the pluripotency is crucial in genome-editing process of hiPSCs. We have compared the several hiPSC culture systems 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 the expression of pluripotency markers, while many of the expanded clones lost the expression of the markers in other systems. In summary, we could establish the highly efficient system for producing the pluripotent hiPSCs with single base mutation using Cas9 RNPs delivered with the optimized ssODNs as donor DNAs in the Cellartis DEF-CS™ culture system.

369. Isoform-Specific Disruption of the BCL11A Transcription Factor Induces γ-Globin but Delays Erythroid Differentiation

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Background: Sickle-cell disease (SCD) and β-thalassemia are common and potentially life-threatening monogenic disorders. Both are caused by defects in β-globin and suitable targets for gene-therapy approaches. Pathology for both disorders is significantly alleviated by elevated levels of the fetal β-like globin, γ-globin, whose expression is curtailed in most adults by the BCL11A transcription factor. BCL11A is essential for the survival of lymphoid cells, and its extra-long (XL) isoform is particularly abundant in erythroid cells. To date no isoform-specific functional assessment of BCL11A has been undertaken. **Aims:** We aimed to target BCL11A for disruption and repair by non-homologous end joining (NHEJ), in order to achieve re-activation of γ-globin expression, i) as a universal therapeutic approach for SCD and β-thalassemia and ii) for isoform-specific functional assessment of BCL11A. Besides a concurrent investigation of the structure-function relationship for the BCL11A transcription factor and its isoforms, a key aim of this study is to evaluate safety and efficacy of isoform-specific disruption of BCL11A-XL for therapeutic derepression of γ-globin in human erythroid β-thalassemic cells. **Methodology/Results:** The RNA-guided endonuclease (RGEN) CRISPR/Cas9 genome editing system was utilized to achieve knockout of the γ-globin repressor BCL11A-XL. Three pairs of short guide RNAs (sgRNAs) were designed to target genomic areas encoding BCL11A-XL. As positive controls for the induction of γ-globin we designed three additional sgRNAs, targeting all BCL11A isoforms, including two targeting the BCL11A translation initiation site. To our knowledge this is the first time this latter approach has been taken to create functional knockouts. After initial evaluation in the HEK293T cell line by transient plasmid transfection, the most active sgRNAs were cloned into lentiviral vectors for further experiments in a HUDEP-2 cell line stably expressing Cas9. Both integrating and non-integrating lentiviral vectors were utilized, the latter towards lower off-target activity and potential translational application. Extending our analyses to functional correction in primary hematopoietic stem/progenitor cells, bicistronic lentiviral vectors encoding both the nuclease and sgRNA component of our RGENs were made and tested in control and patient-derived $CD34^+$ cells. **Conclusion:** Highly efficient sgRNAs have been identified for different BCL11A target sites, with results in HUDEP-2 cells indicating that BCL11A-XL-specific knockouts give high-level γ-globin expression, albeit with intermittent delay in erythroid differentiation. Further experiments in healthy and thalassemic $CD34^+$ cells are underway in order to achieve high-level γ-globin induction in primary erythroid cells and assess potential detrimental effects of BCL11A-XL deactivation, also in the lymphoid compartment, towards functional assessment of BCL11A-XL and in order to gauge the strategy as a translational approach for the therapy of β-globinopathies. * *Shared last authors.*

370. 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 and efficacy 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 mRNA and synthetic crRNA:tracrRNA components. Both experimental strategies involve transplantation of the gene-modified cells in versions of the humanized NSG mouse model (i.e., hu-CD34 or 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.

371. Gesicle-Mediated Delivery of CRISPR/Cas9 for Inactivating the HIV Provirus

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CRISPR/Cas9 gene editing technology has expanded our ability to modify genomic DNA and is being adapted for use as an antiviral therapy in the clinic. For example, CRISPR/Cas9 has been used to specifically target conserved regions of the Human Immunodeficiency Virus (HIV), leading to decreased infectivity and pathogenesis *in vitro* and *ex vivo*. However, the Cas9 and guide RNA (gRNA) effectors

were themselves delivered by viral vectors, which may confer long term expression of the active endonuclease. Thus, the development of alternate strategies to deliver the CRISPR/Cas9 components more transiently could enhance the overall safety of translating CRISPR technology into human therapies. In the current study, we explore the use of a specialized microvesicle (termed "gesicle") as an alternative therapy to treat HIV infection. These particles are incorporated into cells by a mechanism similar to VSV-G pseudotyped lentiviral vectors, but are engineered to deliver a one-time dose of active CRISPR/Cas9 ribonucleoprotein (RNP) complexes, thus bypassing the danger of long-term transgene expression. These RNP complexes are programmed with gRNAs complementary to the sequences within the long terminal repeats (LTRs) of the HIV provirus. We have verified the production of Cas9-containing gesicles using both NanoSight particle analysis and a western blot assay of key gesicle components: mCherry, VSV-G and Cas9. Additionally, we tested their ability to "transduce" and generate mutations within a microglia cell line harboring multiple copies of the HIV provirus (HIV-NanoLuc CHME-5 cells). The application of gesicles results in rapid but transient transfer of Cas9 to the recipient cells, which peaks at 4 hours and is undetectable at 24 hours post treatment. Finally, gesicle delivery of Cas9 + LTR gRNA targeted to the provirus in HIV-nanoLuc CHME-5 cells resulted in site specific mutation of the LTR, reduced the activity of the proviral-encoded luciferase reporter NanoLuc, and inhibited the expression of HIV viral protein Nef. These data suggest that gesicles are a viable alternative approach to deliver CRISPR/Cas9 for targeting the HIV provirus.

372. Manufacturing of Gene-Modified Mouse CAR T-Cells

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The study of the human immune response to cancer is largely performed in a xenograft model in which human tumor and immune cells are transplanted into immunocompromised mice. This model is beneficial for assessing pre-clinical activity and safety of adoptive human T cell therapies. However, as the stromal components of the host are mouse, cellular communication is between mouse and human, and the host is severely immunocompromised, both tumor growth and immune cell responsiveness may not accurately represent disease progression and therapeutic response in immunocompetent mice. To address these issues, we developed a mouse model in which mouse chimeric antigen receptor (CAR) T-cells are adoptively-transferred that target murine antigens. Further, to model our human "off-the-shelf" CAR T-cell based therapies which use TALEN® nuclease targeting the human T cell receptor alpha constant (TRAC) gene to prevent the potential of graft-versus-host disease of the transferred allogeneic CAR T-cells, we eliminate T cell receptor expression in mouse T cells with TALEN® to the mouse TRAC gene which will permit the adoptive transfer of these CAR T-cells to allogeneic hosts. In total, we describe the in vitro culture, transfection and transduction conditions for mouse CAR T-cell manufacturing leading to robust in vitro expansion of functional TALEN®-edited mouse CAR T-cells. Here we use these manufacturing conditions to characterize different CAR constructions and the role of inactivating mutations in various signalling domains in the functional responses of CAR T-cells against a haematological tumor target cell. In conclusion, we describe the conditions for the large-scale production of functional allogeneic mouse CAR T-cells that mimics the human CAR T-cell manufacturing process. Adoptive transfer of these cells will provide a platform to interrogate genetic influences that impact anti-tumor activity and CAR T-cell persistence as well as anti-tumor and on-target/off-tumor toxicities of novel CAR T-cell therapies.

373. Improving Gene Editing with CRISPR/Cas9 System for Recessive Dystrophic Epidermolysis Bullosa Gene Therapy

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The recessive dystrophic epidermolysis bullosa (RDEB) is a rare inherited disease caused by autosomal recessive mutations in the gene *COL7A1* encoding the dermal type VII collagen. The collagen VII is a key component of the skin basal membrane that is anchoring the dermis to the epidermis in order to maintain the skin integrity. There is no cure to date for this disease. One therapeutic approach for RDEB patients is gene therapy by gene editing with the CRISPR/Cas9 system. The Cas9 nuclease, guided by a small RNA (sgRNA), provokes DNA double-stranded breaks at a specific target site which is repaired by one of the cellular DNA repair pathway called homologous recombination (HR). The delivery of a DNA donor template into the cell during HR allows replacement of a mutated gene with a normal allele at the chosen location. The level of HR varies according to the cell type. In order to optimized the rate of HR in all the cells and use this pathway for gene editing, we explored ways to improve the CRISPR/Cas9 system efficacy. For this purpose, we modified the structure of the sgRNA by varying the size of the sequence complementary to the target site and increasing the length of its Cas9 binding sequence. We tested the efficacy of these new sgRNAs on the adeno-associated virus integration site 1 (AAVS1) safe harbor in different human cell types. We used a GFP cassette as a HR donor template to monitor the integration at the AAVS1 site. We successfully increased up to five-fold Cas9 nuclease activity and the level of HR at the AAVS1 site with these upgraded sgRNAs, compared to the non-modified sgRNAs. We then tested these new tools in a gene editing approach for RDEB disease. We measure the HR efficacy in fibroblasts from RDEB patients and obtained 10% of total cell population which integrated the GFP donor at the AAVS1 site. These results confirmed that gene editing using HR pathway is a promising approach of gene therapy for RDEB patients.

374. Combination of Gene and Cell Therapy Increases Therapeutic Anguiogenesis Efficacy

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Cell therapy using autologous progenitors is an intensively developing approach to promote tissue repair and regeneration. We and others have demonstrated potential impact of adipose tissue-derived stromal cells (ADSC) on angiogenic cell therapy for myocardial and limb ischemia. The main benefit of ADSC is that they can be feasibly harvested by a simple, minimally invasive method and also easily cultured. However, efficacy of cell therapy for ischemic diseases is restricted by viability of transplanted cells, which may be exposed to inflamed hypoxic environment of damaged tissue resulting in drastic reduction of their number and, thus, therapeutic effect. Since the initial success of cell therapy for ischemic diseases many attempts have been made to increase its efficacy. One of the promising approaches is a combination of gene and cell therapy by expression of growth factors in ischemic tissues to stimulate transplanted cells' survival and proliferation. The aim of this work was to study the feasibility of combined therapy using ADSC delivery with plasmid-based PDGF-B gene therapy to increase therapeutic angiogenesis efficacy. PDGF-B is important regulator of ADSC function which stimulates their proliferation, migration, inhibits apoptosis of these cells and increases their angiogenic potential. Using a hind limb ischemia model in C57/bl6 mice we assessed the efficacy of combination of ADSC with plasmid based PDGF-B gene therapy for blood flow restoration and angiogenesis in ischemic limb. Intramuscular administration of plasmid encoding PDGF-B gene immediately after hind limb modeling was followed by transcutaneous intramuscular injection of mouse ADSC 5 days later. Blood flow was measured by laser Doppler every 7 days during 21 days; at endpoint animals were sacrificed and skeletal muscle was evaluated for vessel density. Mice injected with PDGF-B plasmid followed by ADSCs transplantation showed significant increase in perfusion compared to plasmid injection or ADSC transplantation alone. These findings were supported by significantly increased CD31+/a-SMA+ vessel density in animals that received combined gene and cell therapy compared to single gene or cell therapy. Obtained results provide a basis for development of gene/cell therapy methods for patients with critical limb ischemia often resulting in amputations especially in diabetic subjects.

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375. Transcriptional Repression for Autosomal Dominant RP

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The RHODOPSIN (RHO) gene is the most common gene involved in autosomal dominant retinitis pigmentosa (adRP). RHO p.P23H and p.P347S mutations account for up to 10% of dominant RP in the US and 5% in Europe. Reducing levels of the toxic products is expected to provide significant benefits. The last decade has witnessed the development of new tools that allow to both the control of gene expression and to engineer genes (genome editing; GE) at their endogenous loci. Among them are Zn-finger (ZF), TALE and, more recently, those based on the CRISPR-associated RNA guided Cas9. In the last years we designed developed and tested transcriptional synthetic transcription factors derived from the eukaryotic zinc finger Cys2-His2 scaffold. To silence RHO gain-of-function mutations in a mutation-independent manner we designed a series of ZF transcriptional repressors specifically targeted to unique human RHO regulatory promoter regions. These ZFs delivered by Adeno-associated virus (AAV) vectors, generated specific reduction of the RHO transcript with remarkable efficiency and specificity, which apparently exceed other platforms (Mussolino C et al., 2011; Botta et al 2016). Built on these results we are currently investigating transcriptional RHO silencing by evaluating the two expression cassettes enclosed in the single vector (DNA-binding repression & replacement). In particular, we generated a set of promoters, which enable the modulation of the repression & replacement to tailor the strategy for the potential clinical translation.

376. Characterization of Targeted Integration with Viral and Non-Viral DNA Donors

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A revolution for the field of genomic therapies is the ability to achieve targeted insertion (TI) of DNA cassettes with high-efficiency and accuracy. Important applications of TI include the delivery of therapeutic transgene expression cargos in known genomic sites that are amenable to robust expression and tolerant to insertions. Use of these so called 'safe harbors', such as the *AAVS1* locus, should increase the uniformity of transgene expression while concomitantly lowering the potential risk associated with semi-random integration of viral vectors (e.g. lentivirus). Furthermore, when endogenous control of gene expression is required, targeted integration of corrected genes/exon at the endogenous site is an attractive strategy. To achieve the ideal level of targeted integration, we have multiple components that are amenable to optimization, from the selection of the CRISPR endonucleases (e.g. WT Cas9, single and dual nickases, Cpf1) to the selection of the donor template (e.g. viral, non-viral, single stranded, double stranded). We will present our work systematically comparing targeted integration

using different CRISPR endonucleases and donor templates in primary T-cell safe harbor sites. We are unravelling the DNA repair mechanisms responsible for mediating integration in response to different type of donors, as well as the optimal donor configuration. Moreover, we assess the efficiency of both on- and off-target integration, a critical consideration while performing donor selection.

377. Develop CRISPR Based Innovative Treatments to Eliminate Persistent Viral Infections

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A latent virus can remain in a host indefinitely, causing persistent infections. Because the viral genome is not fully eradicated by the host's immune system, the virus may reactivate periodically. More serious ramifications of latent viral infection include the possibility of transforming the cell or forcing the cell into uncontrolled division, causing various types of cancer. While antiviral therapies can suppress active viral replication, no existing treatment can effectively eradicate latent infection. During latent infection, the dormant viral genome provides few therapeutic targets other than itself for antiviral drug development, and therefore a cure is lacking for many viral diseases of critical unmet medical need. Agenovir is using state-of-the-art CRISPR/Cas9 and other nucleases designed, engineered, and simulated in silico to disrupt intracellular viral DNA. By disrupting viral DNA, Agenovir's unique strategy makes it possible to treat and eliminate persistent viral reservoirs while leaving host genome untouched. We have generated cell-based efficacy data for several viruses, including HPV and EBV, demonstrating infection-specific cell death following delivery of nucleases to human cells. With an NGS-based off-target detection approach, we have also demonstrated highly specific activity of our drug components.

378. HSPC Expansion Drugs Enhance Gene Editing Efficiency in Long Term Hematopoietic Stem Cells

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Introduction: Gene editing approaches in hematopoietic stem/progenitor cells (HSPCs) offer the ability to inactivate or correct genes involved in a number of infectious and genetic diseases. Previously, we have shown that CCR5-edited HSPCs engraft in autologous nonhuman primates (NHPs), and that T-cells derived from these CCR5-edited HSPCs undergo positive selection in response to infection with simian/human immunodeficiency virus (SHIV). However, the efficiency of gene editing must be increased, especially in long term hematopoietic stem cells (HSCs), in order to provide greater therapeutic advantage to HIV$^+$ patients. Here, we investigated the use of stem cell expansion drugs to increase gene editing efficiency in bulk HSPC and long term HSC subsets.

Methods: A Zinc Finger Nuclease (ZFN) pair that targets a conserved site in the human and macaque sequence was used to target the CCR5 locus in macaque and human HSPCs. CD34-enriched products were collected from NHP bone marrow aspirates and from healthy human donor apheresis products following mobilization. Following enrichment, cells were cultured in defined media in the presence and absence of stem cell expansion drugs, and ZFN mRNA was delivered by electroporation. After editing, cells were sorted on the basis of CD90 and CD45RA expression into CD34$^+$ HSPC subsets including long term HSC. Deep sequencing was used to measure CCR5 editing in each subset.

Results: Human HSPC subsets showed relatively comparable levels of CCR5 editing in bulk HSPCs vs. committed, early and long term HSC subsets. In contrast, NHP HSPCs showed a marked preference for editing in more differentiated HSPC subsets, relative to long term HSCs. Treatment of macaque cells with stem cell expansion drugs led to significant increases in the efficiency of bulk HSPC editing, and long term HSC editing.

Conclusions: We show that pre-treatment of *ex vivo* cultured CD34$^+$ HSPCs with stem cell expansion drugs sensitizes cells to ZFN-dependent editing at the CCR5 locus. We predict that increasing the efficiency of CCR5 editing *ex vivo*, along with key changes to our HSPC culture scheme, will significantly enhance the proportion of gene edited, long-term engrafting cells in autologous hosts. This "next generation" HSPC gene editing approach should offer greater therapeutic efficacy not only for infectious diseases like HIV, but genetic diseases such as hemoglobinopathies and severe combined immunodeficiencies.

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379. CD4-CAR Transduced HSPCs Antagonize Infection in a Nonhuman Primate Model of HIV

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Introduction: Chimeric Antigen Receptor (CAR)-based approaches have demonstrated substantial promise in the treatment of human malignancies. As the fields of cancer immunotherapy and HIV cure have become closely linked, we investigated the use of a well-characterized CAR that exploits the essential interaction between HIV and the helper T-cell marker CD4 (CD4-CAR). Because targeting of infected cells may require a longer time frame than clearance of malignant cells, we further investigated the expression of CD4-CAR from autologous hematopoietic stem and progenitor cells (HSPCs) and their progeny. This approach has the advantage of lifelong expression of the CAR molecule. We hypothesized that HSPC-based CD4-CAR expression would reduce viral replication *in vivo*, and would tightly correlate with viral antigen levels.

Methods: HSPCs were collected from uninfected pigtailed macaques, and transduced with a lentiviral vector expressing CD4-CAR and the

HIV fusion inhibitor mC46. Control animals received an identical vector that encoded a non-functional CD4-CAR molecule. Following autologous transplantation and recovery, animals were infected with Simian/Human Immunodeficiency Virus (SHIV). CD4-CAR marked cells were tracked by flow cytometry and PCR-based assays. SHIV viremia was monitored in peripheral blood and in tissues, both longitudinally and at necropsy. To test whether CD4-CAR marking was antigen-dependent, animals were administered combination antiretroviral therapy (cART) to suppress plasma viremia.

Results: Autologous transplantation with CD4-CAR marked cells was safe, and did not measurably impact hematopoietic recovery. CAR marking was multilineage, and persisted in blood and secondary lymphoid tissues through nearly two years of study. Intriguingly, marking in CD4-CAR animals increased upon SHIV infection, decreased with cART, and increased again following cART withdrawal and viral rebound. In contrast, the marking level of matched control animals fluctuated minimally over an identical course of study. CD4-CAR marked cells displayed virus-specific effector function, protected gut-associated helper T-cells, and limited viral rebound, relative to control animals.

Conclusions: We demonstrate that HSPC-based CAR therapy for HIV infection is safe, results in multilineage CAR-marked cells, persists long term *in vivo*, and is responsive to the level of viral antigen in the periphery. Our approach introduces a "sentinel" that should detect and clear rare reactivated cells as they arise. The ability to introduce a virus-specific CAR into multiple lineages that functionally persist for years is an ideal strategy for eradication of latent HIV, which may recrudesce long after cART withdrawal.

380. Forced Expression of FOXP3 in CD4+ T Cells from FVIII Immunized Mice to Control Inhibitor Development in Hemophilia A Mice

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Therapeutic $CD4^+CD25^+FoxP3^+$ natural regulatory T cells (Treg) can suppress a range of immune functions, including the activation and proliferation of conventional $CD4^+$ T cells. Treg based therapies using ex vivo expanded recipient-derived Tregs are currently in clinical trials as primary or adjunct treatment for allograft rejection and autoimmune disease. We have previously shown that ex vivo expanded polyclonal $CD4^+CD25^+FoxP3^+$ Treg suppressed inhibitory antibody formation against coagulation factors VIII and IX in protein and gene therapies in strain-matched hemophilia A and B mice, including in mice with pre-existing antibodies. Recent studies have demonstrated that adoptive transfer of allospecific Tregs offers greater protection compared to polyclonal Tregs. However, the isolation of extremely rare Treg cell populations for targeted therapy complicates this treatment approach. The transcription factor Forkhead box P3 (FoxP3) is required for suppressive activity of both naturally occurring and acquired Treg. In this study, forced expression of FoxP3 into antigen-specific conventional $CD4^+$ T cells by retroviral gene transfer resulted in a population of antigen-specific cells with phenotypic characteristics of Tregs. Transduced Tregs retained FoxP3 expression and suppressed the proliferation of conventional T cells in both a polyclonal and an antigen specific manner, in vitro. We tested the ability of FoxP3 transduced cells from antigen experienced mice to control the formation of inhibitory antibodies to FVIII protein replacement therapy using an established BALB/c hemophilia A mouse model. Adoptive transfer of purified FoxP3 transduced cells from FVIII immunized mice prevented inhibitory antibody formation in naïve recipients in response to weekly intravenous challenges with FVIII protein. Neither $FoxP3^+$ cells from naïve BALB/c mice or control (empty) vector transduced $CD4^+$ T cells from FVIII immunized mice could significantly control inhibitor formation, suggesting that the prevention of inhibitor responses was antigen specific. We will be testing for reduction/reversal of inhibitory antibodies in mice with established inhibitors by this treatment approach, as well as exploring mechanisms responsible for antigen specific suppression of adaptive immune responses to FVIII by this treatment strategy.

381. Pre-Clinical Pharmacology/Toxicology and Manufacturing Aspects of a Genetically-Modified Hematopoietic Stem Cell Product Candidate for the Treatment of Hemophilia A

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Hemophilia A is a congenital bleeding disorder for which there is no cure, and state of the art therapy consists of bi/tri-weekly intravenous infusions with coagulation factor VIII (fVIII)-containing products. Clinical gene therapies now are underway utilizing liver-directed adeno-associated virus (AAV) delivery of a B domain-deleted human fVIII transgene. Although early clinical data appear positive, at present, this approach remains limited to a subpopulation of patients that do not possess pre-existing immunity to AAV and the duration of therapeutic efficacy has not been defined, but because of the non-integrating nature of AAV the strategy is predicted to be non-curative. A second promising gene therapy platform is *ex vivo* lentiviral vector (LV) transduction and transplantation hematopoietic stem and progenitor cells (HSPCs). This approach is accumulating excellent safety and efficacy data in the setting of several genetic diseases and recently the first such product received regulatory approval. Along these lines, we have been developing a LV-transduced autologous HSPC gene therapy product candidate for hemophilia A. Previously, we identified our lead candidate vector, termed CD68-ET3-LV, as a self-inactivating (SIN), HIV-1-based LV encoding a bioengineered coagulation factor VIII (fVIII) transgene cassette designed for high-level, monocyte lineage-restricted expression. Extensive proof-of-concept characterization was performed using GMP-like vector with transduction and transplantation of murine stem and progenitor cells into hemophilia A mice as well as human $CD34^+$ cell transduction followed by insertion site analysis and transplantation into NSG mice. In preparation for first-in-man testing in a proposed phase 0/pilot study, cGMP CD68-ET3-LV was manufactured, release-tested, and utilized for pharmacology and toxicology product evaluation. A total of 480 mL clinical CD68-ET3-LV was manufactured under cGMP. The resulting LV titer was 4.8+E08 HEK-293T transducing units (TU)/mL and therefore 2.3+E11 total TU were manufactured, which based on the clinical transduction

protocol should be sufficient to transduce 2.3+E09 CD34+ cells and generate enough product to treat approximately 10 adult patients. As a model of autologous CD68-ET3-LV transduced HSPCs, safety and efficacy was evaluated *in vivo* using transplantation of cGMP-CD68-ET3-LV transduced murine HSPCs in the hemophilia A mouse and *in vitro* studies with cGMP-CD68-ET3-LV transduced normal mobilized peripheral blood mononuclear human (mPB) CD34+ cells. Transplant of hemophilia A mice with cGMP-CD68-ET3-LV-HSPC resulted in uniform survival, rapid, multi-lineage hematopoietic reconstitution and induction of therapeutic levels of plasma fVIII activity at low vector copy numbers. Similarly, e*x vivo* transduction of human mPB-CD34+ cells with cGMP-CD68-ET3-LV using an optimized protocol resulted in product containing 1.0 ± 0.5 copies/diploid genome with indistinguishable methylcellulose colony-forming potential compared to mock-transduced cells. Collectively, these data support the *in vivo* pre-clinical safety and efficacy of cGMP-CD68-ET3-LV transduced HSPCs for the treatment of hemophilia A.

382. Lack of WASp Unveils Intrinsic Platelet Defects Sustaining Inflammation and Autoimmunity

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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 thrombocytopenia is one of the main causes of death, the pathogenesis of platelet (PLT) defect is poorly understood. Here, we evaluated the role of WAS protein (WASp) in PLTs in a new conditional mouse model (CoWas) lacking WASp only in the megakaryocytic (MK) lineage.We observed an increased number of MKs and their progenitors both in CoWas mice and complete Was$^{-/-}$ mice (WKO), as well as normal in vitro PLT production by Was$^{-/-}$ MKs. Upon in vivo depletion of PLTs, WKO and CoWas mice are able to restore PLT count with kinetics comparable to wild-type (WT) mice, suggesting no defect in thrombopoiesis. Of note, WASp-deficient PLTs both in WKO and CoWas mice have a shorter half-life and hyper-activated status before and after ADP stimulation and are more prone to apoptosis. We also found that WKO and CoWas mice develop anti-PLT autoantibodies against Was$^{-/-}$ PLTs in line with different proteomic profile. Was$^{-/-}$ PLTs show decreased metabolic activity, increased ubiquitination pathways and Immunoglobulin content. Moreover, Was$^{-/-}$ PLTs release higher amount of soluble CD40L and are able to induce B-cells activation in vitro culture. Finally, WAS patients show increased CD62P and PAC1 expression at steady state and higher sCD40L plasma levels. The activation profile of human PLTs improves after LV-gene therapy treatment, in all the patients, especially in those with a follow up longer than two years.

383. TALEN Induced Fetal Hemoglobin Expression in Erythroid Progeny of Human CD34 Cells

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Hemoglobinopathies including sickle cell disease (SCD) and β-thalassemia are the most common single-gene disorders in the world and represent a major global public health concern. The unifying principle of this heterogeneous mix of gene mutations is the decreased production of wild type hemoglobin molecules either due to structural defects in the case of SCD or insufficient production of β-globin subunits. Hemoglobinopathy patients with gene variants that induce expression of fetal hemoglobin often exhibit a less severe phenotype. Recent evidence has demonstrated that the in vitro induction of a previously described 13bp deletion in the promoter of the Aγ globin gene can mimic the induction of fetal hemoglobin seen in patients with this mutation. As temporary expression of endonuclease is a key element of a clinically viable editing technique, we have generated a set of TALENs targeting the 13bp deletion site that are transfected as mRNA. In human CD34 cells, expression of the TALEN pair results in INDEL generation at both the Aγ and Gγ globin loci confirmed by colony sequencing, T7 analysis and ddPCR. INDEL generation was more efficient in the Aγ globin locus (~33%) compared to the Gγ globin locus (~8%). Compared to control cells, edited CD34+ cells placed in erythroid differentiation media result in an increased expression of fetal hemoglobin with a near doubling in the frequency of 'F-Cells' (γ globin expressing cells) by flow cytometry and a three fold increase in γ globin protein detected by HPLC. These findings support a model in which TALEN-induced targeted INDELs in the γ globin promoter of hematopoietic progenitor cells de-repress fetal hemoglobin in erythroid progeny. TALEN edited CD34 cells have also been transplanted into W41 mice, a strain demonstrated to be more permissive of human erythropoiesis, and are capable of engraftment. AAV transfected homologous recombination templates have also been generated with a goal of further manipulating hemoglobin expression and allowing for the selection of edited cells. A simple repair template that constitutively expresses GFP has been successfully integrated at the target site (approximately 27% efficiency) resulting in erythroid and myeloid methylcellulose progenitor colonies detectable by fluorescence microscopy. Other repair templates that either introduce the specific 13bp deletion or introduce elements of the β globin promoter result in higher rates of HbF expression in GFP+ cells. Continued efforts are underway to optimize repair template designs, editing efficiency and selection strategies to prepare this approach for clinical applications.

384. Gene Edited Hematopoietic Stem/ Progenitor Cells for Treating Sickle Cell Disease

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Introduction: Sickle cell disease (SCD) is an inherited blood disorder associated with a debilitating chronic illness. SCD is caused by a point mutation in the β-globin (*HBB*) gene. A single nucleotide substitution converts a glutamic acid to a valine that leads to the production of sickle hemoglobin (HbS), which impairs the function of red blood cells (RBCs). In this study, we show that targeted genome editing potentially provides a permanent cure for SCD by correcting the sickle mutation in *HBB* in hematopoietic stem and progenitor cells (HSPCs) for autologous transplantation. **Materials and Methods:** Using nucleofection, we delivered *Streptococcus pyogenes (Spy)* Cas9 protein and guide RNA as a Ribonucleoprotein complex in conjunction with a single-stranded DNA donor template to correct the sickle mutation in *HBB* of HSPCs from SCD patients, which were then cultured in an erythroid-differentiation medium and assayed as both single-cell clones and in bulk culture. The off-target activity of *HBB*-specific gRNAs in HSPCs was assayed using both informed bioinformatics and unbiased genome-wide analysis techniques. **Results and Discussion:** Using a highly optimized genome-editing system, we achieved up to 39% homology directed repair (HDR) mediated gene-correction rates in HSPCs. The frequency of HDR alleles persisted over the course of differentiation, and up to 43% of erythroid colonies were either homozygous or heterozygous for gene-corrected alleles. Normal erythroid development and total hemoglobin levels were observed in edited HSPCs. Erythrocytes derived from treated cells expressed hemoglobin A (HbA) and had increased levels of fetal hemoglobin (HbF); furthermore, they showed improved anti-sickling properties in a hypoxia assay, demonstrating a direct genotype-phenotype relationship. We also observed a strong correlation between the frequencies of non-homologous end joining (NHEJ) at the target site and increases in HbF levels compared to control cells, as indicated by a γ- to α- subunit ratio 3 times higher in the treated sample, suggesting that *HBB* disruption induces HbF by upregulating *HBG*, which compensates for the shortage of beta subunits from the *HBB* knockout. This is especially encouraging because this compensation may ameliorate toxicity of excess alpha globin in biallelic *HBB* knockout cells, thereby improving the therapeutic efficacy of our approach. Ongoing studies include xeno-transplantation into NOD/SCID mice to measure the ability of the gene edited HSPCs to reconstitute hematopoiesis *in vivo* and provide further characterization of the mechanism and therapeutic potential of HbF induction. **Conclusions:** Our results demonstrate that CRISPR-Cas9 based genome editing has the potential to provide a permanent cure for SCD by correcting the sickle mutation and inducing HbF expression in HSPCs from SCD patients. Additional studies are needed to better understand the induction of HbF and demonstrate *in vivo* engraftment in order to facilitate the clinical translation of our genome editing based strategies for treating SCD.

385. T Cells Can Serve as a Vehicle for Factor IX Gene Replacement

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Systemic gene transfer by viral delivery is a promising form of treatment for the hemophilias. However, concerns remain about in vivo delivery which may limit widespread adoption, including pre-existing immunity in some patients and the potential for oncogenesis. An ex vivo approach could help to solve these limitations, but the cell type used must be engraftable and capable of producing active Factor VIII or IX.

Recently, gene-engineered T cells have been shown to be a promising treatment for some types of cancers. Human T cells can be easily obtained from peripheral blood and transduced with retroviral or lentiviral vectors encoding a chimeric antigen receptor (CAR) gene targeted to a specific cell surface proteins. The CAR T cells are then transferred back in to patients, and can eliminate tumor cells bearing the targeted protein. CAR T cells have been found to be safe and efficacious, and can persist for long periods of time.

Based on the success of CAR T cells, we reasoned that T cells might also serve as a cellular vehicle for coagulation factor gene transfer to treat hemophilia A or B. To test our hypothesis, we generated lentiviral vectors encoding FIX with a hyperactivating FIX-R338L mutation (LV-FIX-Padua). LV-FIX-Padua efficiently transduced over 80% of primary human T cells. Importantly, we detected secretion of FIX antigen (4-7% of normal) and FIX activity (20-30% of normal) in the supernatant, demonstrating that T cells are capable of secreting active FIX. We then injected the FIX-Padua-expressing human T cells in to immune deficient mice (NSG), and observed therapeutic levels of FIX in the serum (20-40% of normal human FIX levels) at 6 weeks of analysis. Similarly, transfer of FIX-expressing murine T cells in to C57BL/6 mice resulted in 2-20% of normal human FIX levels in the serum. Studies are now underway to evaluate the approach in hemophilia B mice.

These studies suggest that T cells can serve as a vehicle for FIX gene replacement therapy, and could utilize the existing infrastructure and safety record established for CAR therapy to facilitate adoption to the clinic.

386. Inducible KLF1 Gene Expression Successfully Ameliorates Hemoglobin Switching in Erythroid Cells Derived from Congenital Dyserythropoietic Anemia (CDA) Patient Specific iPS Cells

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The congenital dyserythropoietic anemias (CDAs) are inherited red blood cell disorders representing ineffective erythropoiesis and dyserythropoietic changes in the bone marrow. We recently diagnosed

a female patient with undiagnosed congenital anemia as type IV CDA caused by a heterozygous missense mutation of the erythroid-specific transcription factor, *KLF1*; c.973G>A, p. E325K. To assess the possibility of gene therapy for type IV CDA, we generated induced pluripotent stem cells (iPSCs) from peripheral blood T lymphocytes of the patient (CDA-iPSCs), and examined transgenic expression of normal human *KLF1* in erythroblast derived from CDA-iPSCs. Tet-on inducible *KLF1* expression cassette was divided into two portions, and inserted in loci of adeno-associated virus site 1 (AAVS1) of CDA-iPSCs by electroporation delivery of transcription activator-like effector nuclease (TALEN) pairs with donor vectors as shown in Figure 1. Successfully targeted cells were selected by the acquisition of antibiotic resistance. Insertion of inducible gene expression cassettes were confirmed by PCR analysis and sequencing analysis of genome DNA. Transgene expression level reached a plateau at 48 hours in the presence of doxycycline (Dox) in culture media, and 1,000 ng/ml Dox was enough to induce high expression of transgene. Dox treatment in erythroblast derived from targeted CDA-iPSC achieved approximately 5- to 10-fold increase in *KLF1* mRNA with endogenous expression level and resulted in increase of *KLF1* target genes. Among them, *beta-globin*, which encodes adult-type hemoglobin and is one of the most popular *KLF1* target gene, was increased more than 100-fold from that of non-treated control. Our data suggested that transduction of normal human *KLF1* cDNA in human hematopoietic stem cells would be an effective therapeutic strategy in type IV CDA. We also propose that our model of iPSCs provides insights on understanding the mechanisms of type IV CDA and it will be a useful tool for assessing possibilities of novel treatments for the rare congenital anemia.

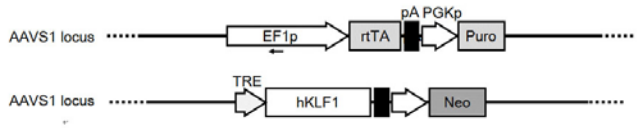

Figure 1 Inducible KLF1 expression cassette

387. Intraosseous Delivery of Lentiviral Virus Successfully Produces Human Platelet-Specific Factor VIII in Humanized NSG Mice

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Hemophilia A (HemA) with a deficiency of functional plasma factor VIII (FVIII) is an ideal disease model for *in vivo* gene therapy to attain long-term therapeutic level of FVIII expression. We have successfully developed a new clinically translatable strategy for treating HemA by using intraosseous (IO) delivery of lentiviral vectors (LVs) carrying a human FVIII/N6 transgene driven by a platelet-specific Gp1bα promoter (G-F8-LV) into HemA mice without preconditioning treatment. FVIII stored in platelet α-granules partially corrected the bleeding phenotype over five months in immune-competent HemA mice with or without pre-existing anti-FVIII inhibitors. In this study, we aimed at establishing proof-of-principal translational research models for human applications of this novel strategy in human cells and humanized mice. Firstly, we evaluate if high level FVIII gene expression can be achieved in human megakaryocytes (Megs) differentiated from G-F8-LV transduced human $CD34^+$ cells *in vitro*. Human $CD34^+$-enriched cells from G-CSF mobilized donors were cultured and upon differentiation using cytokine cocktail (CC110) for expansion of hematopoietic stem cells (HSCs) or StemSpan™ Meg expansion supplement (MEG), significant higher numbers of $CD41a^+$ Megs were generated in cells supplemented with MEG media compared with cells with CC110. Interestingly, cells evaluated on day 13 not only showed higher percentage of $CD41a^+$ Megs, but also had more late-stage differentiated Megs ($CD41a^+CD42b^+$) compared with cells on day 7, indicating that human $CD34^+$ cell were successfully propagated and differentiated into Megs in culture. Furthermore, we successfully transduced human $CD34^+$ cells using MND-GFP-LV driven by the ubiquitous MND promoter and G-GFP-LV (MOI=5), respectively, and the GFP^+ cells were maintained upon adding CC110 or MEG media. Subsequently, human $CD34^+$ cells were transduced with G-F8-LV, and then stimulated into Megs by MEG media. Flow cytometry analysis confirmed that FVIII transgene was expressed in $CD41a^+$ Megs. The transduced cells will be harvested and FVIII expression levels will be examined by FVIII-specific ELISA. Viral copy numbers and vial integration sites in transduced cells will be evaluated by qPCR and LAM-PCR, respectively. We anticipate that following differentiation, we will obtain higher levels of FVIII expression in MEG-mediated differentiated cells than CC110-mediated differentiated cells. In addition, higher $FVIII^+$ cells will be observed in late-stage $CD41a^+CD42b^+$ differentiated Megs. Secondly, we investigate FVIII expression following IO delivery of LVs in humanized NSG mice. Humanized NSG mice were created by retro orbital injection of human $CD34^+$ cells into busulfan (25 mg/kg) pretreated NSG mice and ~40-60% engraftment of human $CD34^+$ cells were achieved. IO infusion of G-GFP-LV or G-F8-LV is then carried out in the humanized mice several weeks later. Human platelets will be isolated from treated mice and assayed for FVIII levels and functionality. Three months later, LV-transduced human HSCs are characterized by isolating bone marrow cells from the treated mice for colony forming cell assay. Taken together, IO infusion of G-F8-LVs into humanized NSG mice could be a valuable experimental test model for translational research for *in vivo* gene therapy of hemophilia A.

388. Development of an In Vitro Biopotency Assay for FIX AAV Gene Therapy Vectors in Human Liver Cells That Correlates with In Vivo Data from FIX Knockout Mice

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Introduction and Objective: A criterion for release of a FIX gene therapy vector is its biopotency, which is usually determined in FIX ko mice after administration into the tail vein & measurement of FIX activity in plasma. To circumvent laborious in vivo assays using animals, we developed an in vitro method using human HepG2 liver cells & compared the results with biopotency data generated in parallel

in mice. **Methods:** In the in vitro assay, AAV8-FIX vector preparations are used to infect preconditioned human HepG2 liver cells at a defined multiplicity of infection (MOI). Subsequently, FIX is secreted into the culture medium & FIX activity is determined by chromogenic assay. Each assay run includes a standard curve of purified AAV-FIX vector material using MOIs in a defined range. In parallel, the same vector preparations were administered intravenously into FIX ko mice & FIX activity was measured two weeks later. The data sets from both assays were analyzed using various statistical approaches. Regression techniques were used to test for correlation between the two methods. Variation decomposition analyses were done to compare measurement variation between the two test methods, & to assess the ability of the test system to differentiate between different samples. **Results:** The in vitro assay allows measurement of AAV8-FIX preparations in a dose-dependent, linear manner. Comparison of the biopotency of 25 purified FIX vector preparations in vitro & in vivo did not indicate a significant mismatch, as shown by analysis of variance. The two methods demonstrated excellent matching behavior, meaning that a low (or high) biopotency in the mouse assay corresponded to a low (high) biopotency in the cell-based assay & thus, measurement averages for both methods were highly correlated. The in vitro method showed less and more uniform variation in residuals than the in vivo test. **Conclusions:** Results obtained from the in vitro biopotency assay correlated significantly with those from the in vivo test. The cell culture-based release assay showed less variation than the mouse-based method, indicating that a switch to this assay may be feasible.

389. *In Vitro* Effects of CAR T Cells Targeted Against the HCMV Glycoprotein B: TCR-Independent Killer T Cells for Transplanted Patients

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Introduction: Patients treated with hematopoietic stem cell transplantation are faced with numerous potential life threats in the first months. Latent cytomegalovirus (HCMV) infection in seropositive patients is generally asymptomatic, unless the virus reactivates into a lytic phase when grafts cannot provide sufficient immune control. This significantly worsens prognosis. Standard treatment relies on antiviral drugs but more efficient and less toxic options are needed. Thus, cellular therapies against HCMV have been explored. Because of HLA down-regulation on infected cells, HLA-dependency of target recognition is problematic for adoptive transfer of HCMV-reactive T cells and for T cell receptor engineering approaches. We have therefore developed chimeric antigen receptors (CARs) that target HCMV-infected cells in a TCR-independent manner. We have selected the glycoprotein B (gB) as the antigenic target, a late HCMV protein expressed for several weeks on latently infected cells after reactivation. **Methods:** To create gB-CARs, we used the high-affinity monoclonal antibody SM5-1 that binds to a non-glycosylated conserved gB epitope, and is capable of neutralizing infectious HCMV particles. The single chain variable fragments (scFvs) of SM5-1 in both $v_H \rightarrow v_L$ and $v_L \rightarrow v_H$ configurations were generated and fused with CAR backbones containing a C_H3 IgG Fc spacer and CD3ζ and CD28 or 4-1BB signalling domains. We used γ-retroviral vectors encoding CARs to transduce peripheral blood mononuclear cells (PBMC) activated with CD3 and CD28 antibodies in the presence of interleukins 7 and 15. After transduction, CARs were stably expressed in 80 to 95% of T cells. **Results:** To test target recognition, gB-CAR T cells (gBCARTs) were co-cultured with 293T cells highly expressing gB. 293T cell death was determined by flow cytometry using 7-AAD staining. Specific killing of 30 to 40% of gB^+ compared to w.t. 293T was detected after 2 days at an effector to target ratio of 3:1 using CD19CAR T cells and non-transduced T cells as controls. Cytotoxicity was more pronounced in gBCARTs with the scFv in $v_L \rightarrow v_H$ orientation (up to 5% difference) and was similar in gBCARTs with CD28 or 4-1BB costimulatory domains. gBCARTs were also cytotoxic against HepG2 cells infected with a laboratory HCMV strain (TB40E) although the analysis was difficult due to significant alloreactive T cell activation and high background target cell killing by control T cells. To overcome this problem, an entirely autologous assay was established. PBMCs from HCMV-seronegative donors were infected with a luciferase-expressing strain of HCMV (TB40E-gLuc) in the presence of G-CSF and co-cultured with gBCARTs produced from the same donors. gBCARTs cleared HCMV-immediate early antigen 1 positive cells as measured by immune fluorescence and resulted in reduction of gLuc detection. We are currently developing a quantitative flow cytometry-based assay to compare the kinetics and efficacy of various CAR designs to kill HCMV-infected cells. **Conclusions:** We have developed gBCARTs that elicit an *in vitro* killing effect against HCMV-infected cells. We are currently optimizing vector and CAR design in order to next validate gBCARTs in an *in vivo* humanized mouse model of HCMV-reactivation already established in our laboratory.

390. Hemophilia B Gene Therapy Via CRISPR-Cas9 Targeted Integration of the Factor IX Gene

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A core objective of gene therapy for hemophilia is the establishment of long term therapeutic gene expression of coagulation factors. Considerable progress has been made using Adeno-Associated Virus (AAV) vectors for effective treatment of hemophilia in patients, representing a milestone in gene therapy for serum deficiency disorders. Yet, these same studies have highlighted the gradual reduction of gene expression impacting therapeutic benefits. One supposition posits that loss of episomal vector genomes is a factor partially responsible for the transient nature of expression. A potential solution to this obstacle is targeted integration of transgenes to provide extended expression, as has been achieved in other contexts with retro- and lenti-viral gene therapy. Herein, we hypothesize adenoviral-mediated gene therapy can

establish long term therapeutic FIX expression following homology directed repair using a CRISPR/Cas9-based system targeted to the 'safe harbor' *ROSA26* locus in murine a model of Hemophilia B. In order to test this concept we have utilized adenovirus owing to its key advantages including large cloning capacity, transient expression of gene editing machinery, and lack of dependency on helper viruses for production. In addition, adenovirus has been utilized as a successful delivery platform for CRISPR/Cas9 strategies and has been shown to be a preferential vector for donor DNA, which may be critical for insertion of larger transgenes. To date, we have edited the *ROSA26* locus in murine liver cells by inducing mutations resultant from non-homologous end joining (NHEJ) as well as successfully generating on-target insertions of our 2Kb reporter cassette of the GFP transgene driven by the EF1α promoter. Secondly we have shown in vivo utility of both NHEJ activity and obtained preliminary data on rates of targeted insertion of our reporter cassette; this integration was achieved through co-injection of a vector containing GFP reporter cassette, flanked by *ROSA26* specific sequences, and a vector encoding a *ROSA26* specific guideRNA and the Cas9 endonuclease. Currently, we are tracking serum levels of episomal and integrating A1AT expression in vivo using a donor vector isogenic to our GFP reporter donor to determine if integration affords extended gene expression. In the final portion of our study, we will integrate FIX at the *ROSA26* locus in the FIX KO mouse strain, which faithfully recapitulates the human disease, to test levels of FIX expression, therapeutic FIX activity, and improved coagulation phenotypes while monitoring for vector mediated toxicity. If successful this study will represent the feasibleness of CRISPR/Cas9 system for the targeted integration of therapeutic genes in post embryonic somatic tissue using adenovirus while providing further information on the utility of CRISPR/Cas9 application to the widest breadth of serum deficiency disorders.

391. Comprehensive Systematic Assessment of Immune Repertoire Analysis Methods

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High-throughput sequencing has broadened the possibility to dissect the immune repertoire at a higher resolution to deepen our understanding of the adaptive immune system. Most significant insights can be gained relative to various states as cancer, autoimmune conditions, infection and aging process. Additionally, it can enhance our ability to uncover the underlying mechanisms of immunity in health and disease conditions. With further progress in sequencing technologies higher amount of data is being generated, which requires sophisticated analysis methods. Various tools have been developed so far for immunosequencing analyses and in this aspect progress is currently underway. The major analysis aim is to unravel the diversity of the immune system and perform composition profiling to obtain clinically relevant information. In T cell receptor analyses the general strategy includes steps to determine gene segments by aligning sequences to the reference set, clonotypes identification, detection of CDR3 region and their abundance estimation. To provide a practical comparison of the computational methods for immune repertoire analyses, we have conducted an in-depth and systematic comparative study of eight available methods. We have employed numerous in silico and experimental datasets to perform thorough assessment of each approach in view of various analysis factors. Moreover, a clonal plane analysis strategy is used to perform clonality analysis of samples under investigation. In addition, we describe in detail the substantial effects of choice of analysis method on interpretation and outcome. Our study will enable researchers in this filed to select an optimal analysis method and in this regard will provide basic evaluation based guidelines.

Immunological Aspects of Gene Therapy and Vaccines II

392. DNA-Monoclonal Antibody (DMAb) Gene Delivery Against Ebola Virus Disease (EVD), an *In Vivo* DNA Vectored Approach for Achieving Sustained, Transient Serum Levels of Protective IgG

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Monoclonal antibodies (mAbs) are an important treatment approach for several diseases, prevention of graft rejection, and represent potential strategy for treatment of emerging infections in humans. However, delivery of bioprocessed IgG mAb targeting infectious diseases is met with significant hurdles including development and manufacturing costs, and the requirement for several high-dose administrations (mg/kg), especially during a possible outbreak such as Ebola virus disease (EVD) where time is of the essence. We recently described the novel engineering and development of synthetic plasmid DNA-vector encoded antibodies (DMAbs) as an alternative approach for *in vivo* immunoglobulin heavy and light chain gene delivery. DMAbs are administered by *in vivo* transient transfection of skeletal muscle mediated by electroporation, employing the cells as biological factories to secrete a functional antibody at detectable levels in systemic circulation. DMAbs can be designed to encode a range of mAb isotypes and synthetic bispecific isoforms. Several mAb clones targeting the Ebola virus glycoprotein (GP) have demonstrated successful protection in animal studies with lethal Ebola virus infection and have been associated with favourable recovery in confirmed human EVD patients receiving the anti-GP mAb cocktail, ZMapp. Using this engineered gene delivery platform, we evaluated >30 DMAbs encoding anti-Ebola virus

GP mAbs that target the GP glycan cap, fusion loop, chalice base, and HR2 region. BALB/c mice were administered individual DMAbs alone or in combination. EVD DMAbs were detected in mouse serum for >100 days, with several candidates reaching 20-80μg/mL C_{max} serum IgG levels. Two EVD DMAbs prevented mortality and morbidity in 100% of animals following a stringent 1000LD50 dose of mouse-adapted Zaire Ebola virus on day 28 post-DMAb administration. Furthermore, 40% of animals survived lethal challenge administered 82 days following DMAb administration, demonstrating long-term systemic circulation of muscle-produced DMAb-IgG. Ongoing studies are evaluating EVD DMAb expression in guinea pigs and non-human primates. DNA-monoclonal antibody delivery is a transformative technology that enables long-term transient IgG expression, with the possibility for repeat DMAb administration. The DMAb platform expands the use of DNA vector technology for gene delivery and other transient gene based therapy applications such as replacement protein or enzyme delivery.

393. Development of Recombinant Adeno-Associated Viral Vector (rAAV) for Passive Immunisation Against Ebola

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The 2014-2016 Ebola outbreak in West Africa was the largest in history leading to 28,639 cases, of which 40% were fatal. Better treatments and effective vaccines against Ebola virus are needed to prevent future recurrence. Multiple, novel, treatment options were assessed during the outbreak, including ZMapp™ monoclonal antibody therapy, which showed promising results in some patients. However, widespread adoption of this approach is hindered by high costs of monoclonal antibody manufacturing and the relatively short half-life of antibody in the blood circulation. To circumvent such limitations, we propose to utilise recombinant adeno-associated virus (rAAV) delivered to the muscle to express the therapeutic monoclonal antibodies. A single intramuscular dose of rAAV encoding lipoprotein lipase has shown clinical efficacy in patients for at least 6 years demonstrating persistent transgene expression, together with a well-established safety profile. During an initial proof-of-principle study, we showed that a single dose of rAAV2/8 carrying secretory reporter gene (*Gaussia* luciferase (GLux)) leads to abundant expression of GLux at day 7 post administration, which increases and persisted in the blood circulation for at least 28 days (20,865 RLU/μl, 43,420 RLU/μl and 6220,000 RLU/μl; $p<0.01$, respectively for 1E9, 1E10 or 1E11 Genome Copies). We then produced rAAV2/8 encoding KZ52 antibody, the first human anti-Ebola (*Zaire* strain) neutralising antibody, isolated from a recovered patient during the 1995 Kikwit Ebola outbreak. A single intramuscular dose of rAAV2/8 encoding KZ52 leads to expression of high human IgG (256.36 μg/ml) in the mouse serum at day 28 post administration, which is ~100-fold higher than the EC_{90} required for *in vitro* neutralisation of the wild-type Ebola virus (J Virol 73:6024-6030,1999). In terms of functioning titre in the serum, we detected high serum binding and neutralising antibody titre against an Ebola (*Zaire*) glycoprotein pseudotyped influenza virus (EC_{50}: 1:7000 & 1:4356, respectively) in BALB/c mice following a single dose of 1E10 or 1E11 Genome Copies of rAAV2/8 expressing KZ52. To test its efficacy in preventing Ebola infections, we are currently undertaking a protection study against Ebola in the guinea pig challenge model. Together, these preliminary data support the generation of rAAV vectors carrying a cocktail of novel antibodies isolated from vaccinated donors for maximum clinical efficacy. In the event of future outbreaks, matched rAAV cocktails which were produced in advance, lyophilised and stockpiled can be used to provide timely prophylaxis among healthcare workers.

394. Influence of Pre-Existing Anti-Capsid Neutralizing and Binding Antibodies on AAV-Mediated Liver Transduction

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The prevalence of pre-existing immunity to adeno-associated virus (AAV) in the human population greatly hinders the global reach of emerging gene-based therapies exploiting this vector platform, particularly when systemic administration is warranted. Beyond the notion of neutralization, the dynamic consequences of such immunity in the form of anti-capsid immunoglobulins on transduction efficiency, vector biodistribution and vector immunogenicity remain largely elusive. Upon screening human serum samples for the presence of anti-AAV8 antibodies, we identified adult donors who harbor circulating anti-capsid binding antibodies devoid of neutralizing activity (BAbs), which were assessed for the ability to influence AAV vector-mediated liver transduction, in comparison to neutralizing antibodies (NAbs). An in vitro virus uptake assay employing imaging flow cytometry revealed that internalization of AAV8 capsid in a murine hepatocyte cell line was detectable both in the presence of anti-AAV8 BAbs and NAbs. However, transgene expression was detectable only when transduction was performed in the presence of BAbs. Animal studies demonstrated that anti-capsid NAbs mediate rapid clearance of systemically-delivered vector from the bloodstream and sequester it to both the parenchymal and non-parenchymal compartment of the liver, leading to lack of transgene expression. Conversely, in mice passively immunized with BAbs prior to intravenous delivery of AAV8 vector, we observed enhanced vector persistence in the circulation and transduction capacity in the liver and skeletal muscle, compared to PBS-injected animals and mice immunized with serum derived from individuals naïve to AAV. The humoral immune response mounted against AAV8 capsid, in the form of vector-specific immunoglobulins, following gene transfer was dampened in mice with pre-existing circulating BAbs and NAbs, compared to that observed in PBS control mice. These results establish the clinical significance of non-neutralizing antibodies directed against the AAV capsid in influencing measures of systemic gene transfer and vector immunogenicity.

395. Successful Repeated Hepatic Gene Delivery in Non-Human Primates Achieved with AAV5 by Use of Immune Adsorption

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A major challenge in AAV-based gene therapy is the presence of circulating neutralizing antibodies (NABs) against AAV vector capsids that may prevent successful transduction of the target cells. NABs can be present in patient's blood prior to AAV treatment due to naturally acquired infections with the wild type AAV virus (pre-existing NABs). Anti-AAV NABs are also raised after first administration of AAV in the course of gene transfer treatment. There is a need to develop strategies that would permit AAV-mediated gene delivery to patients with pre-existing anti-AAV NABs, including patients previously treated with AAV-mediated gene transfer who might experience over time a decrease in therapeutic protein expression due to the natural turnover of transduced cells or insufficiently high transduction.

To address those issues, we explored the feasibility of using an extracorporeal immune-adsorption procedure for repeated, liver-targeted gene delivery in non-human primates (NHPs). NHPs (3 animals per group, 2 groups) tested negative for the presence of anti-AAV serotype 5 NABs, were first injected intravenously (dose of $1x10^{13}$gc/kg) with AAV5-hSEAP. Four months after the first AAV injection, all the animals were re-injected with AAV5-hFIX (dose of $3x10^{13}$gc/kg). Three of the animals were submitted beforehand to immune-adsorption. The levels of anti-AAV5 neutralizing antibodies (NABs) were determined immediately before and after the procedure. The transduction efficacy of both AAV5-hSEAP and AAV5-hFIX were assessed by measuring the levels of circulating hSEAP and hFIX proteins as well as the amounts of AAV vector DNA present in the liver at sacrifice (three weeks after the second AAV injection).

After 1 session (3 cycles) of immune-adsorption, the levels of NABs were decreased by a mean factor of 12. Sequential AAV-based gene delivery with AAV5 after immune adsorption proved to be successful an effective transduction was achieved for the two reporter transgenes (hSEAP and hFIX) used in the study. Furthermore, the hFIX levels obtained after re-administration were in the same range of the hFIX levels obtained after primary administration of AAV5-hFIX. In contrast, the re-administration of AAV5-hSEAP followed by AAV5-hFIX without immune adsorption was unsuccessful due to the total inhibition of secondary AAV5 transduction by anti-AAV5 NABs.

In summary, our data demonstrates that the use of an immune adsorption procedure enables successful re-administration of an AAV5-based gene transfer in NHPs.

396. Improved In Vitro Assay to Assess Human Serum Neutralization of AAV Vectors Yields Cell Line-Dependent Results

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Adeno-associated virus (AAV) vectors have served as a promising delivery platform for gene therapy in a wide range of clinical applications. A consideration in vector choice is pre-existing AAV vector-neutralizing activity (VNA) in serum of subjects in target populations. Inability to detect VNA in potential subjects due to lack of robust screening assays may result in inclusion of patients in clinical trials who may not respond to an AAV-delivered therapeutic only due to reduction of transduction efficiency by serum VNA. This circumstance is anticipated to impact efficacy in clinical trials. VNA is typically assessed in vitro by measuring serum inhibition of cell line transduction by AAV vectors expressing reporter genes. Adequate signal at low AAV multiplicity of infection (MOI) permits detection of low-level VNA by improving signal-to-noise ratio in detection of reporter expression. Here, we present an improved VNA assay. We designed an AAV vector that accomplishes high expression levels using a CMV promoter, an HBB intron, a WPRE 3' element, and a Nanoluciferase® reporter gene encoding a high-brightness luciferase variant. Selected cell lines previously reported as useful in the VNA assay were tested, including U-87 MG, HeLa, HeLaRC32, HT-1080, HepG2, 293T, COS1, and COS7; MOI from 150 to 5000 provided acceptable signals. This optimization resulted in requirement for up to 600-fold lower MOI than preliminary versions of our assay. Most published AAV neutralization assays rely on an IC50 endpoint to determine positivity. This definition lacks the statistical justification recommended by FDA for such assays, and requires highly precise data for an adequate 4-parameter fit for accurate calculation. In our assay, we have implemented a statistical cutpoint determination based on FDA draft guidance for immunogenicity assays. The cutpoint is defined to eliminate 95% of negative sera, yielding 5% false-positives. Provisional cutpoints in various cell lines were established based on relatively small numbers of sera and used to identify high-positive sera for use as controls while sera with close to no detectable VNA were pooled as provisional negative control. In the final version of the assay, all serum dilutions are performed in the final negative control pool to help distinguish VNA from matrix effects. Further development will include analysis of large numbers of negative human sera (>50) to establish true cutpoints. Further refinements to increase differences between signals from sera positive and negative for AAV VNA are under investigation. Surprisingly, some sera showed discordant VNA profiles in different cell lines, with high VNA in some cells, but very low or undetectable VNA in others, suggesting that VNA is cell line-dependent. This disparity exists in ~5% of the sera tested and involves multiple cell lines; its origin is under investigation. Since different cell lines have been used in VNA assays in the literature to support various clinical trials using AAV vectors, observation of cell line-dependent neutralization results calls for caution in interpreting data obtained from in vitro VNA assays and extrapolation/prediction of responses to AAV vector therapy, and underscores the need for more robust VNA screening assays.

397. Immunomodulatory Drug Fingolimod (FTY-720) Inhibits Distal T Cell Receptor (TCR) Signaling in Human T Cells

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Introduction: Fingolimod (FTY-720) is a FDA approved immunomodulatory drug used to treat relapsing forms of multiple sclerosis in adults. FTY-720 down regulates S1P receptor resulting in lymphocyte sequestration in the lymph nodes. Recent studies suggest FTY-720 directly inhibits T cell activation (TCA) independently of the S1P pathway; however, the mechanism of inhibition is not completely understood. Understanding the mechanism of FTY-720 mediated inhibition of TCA may provide novel insights into its immunosuppressive effect, and strategies to develop novel FTY-720 based immunomodulatory therapies. Here, we characterized the effects of FTY-720 on T cell receptor (TCR) signaling pathways. **Methods**: Jurkat T cells and primary human T cells obtained from healthy blood donors were treated with FTY-720. Following stimulation with anti-CD3, activation of proximal T cell receptor (TCR) signaling was assessed by measuring phosphorylation of Lck, ZAP-70 and LAT by immunoblots. Following stimulation with PMA/Ionomycin (PI), activation of distal TCR signaling was assessed by measuring IL-2 and IFN-γ release (ELISA). NFAT nuclear translocation was measured by immunoblots and confocal microscopy and NFAT-dependent promoter activity was assessed by luciferase assays. **Results:** FTY-720 did not inhibit proximal TCR signaling; however, it significantly inhibited PI induced distal TCR signaling in a dose-dependent manner as measured by IL-2 and IFN- γ release. FTY-720 did not inhibit NFAT activation and nuclear translocation. The inhibitory effect of FTY-720 on distal TCR signaling was partially rescued by IPA3 (p21 activated kinase 1, PAK-1 inhibitor). IPA3 alone did not interfere with PI induced IL-2 release; however, inhibited anti-CD3/CD28 induced IL-2 release. **Conclusions:** FTY-720 inhibits distal but not proximal TCR signaling in human T cells. The effect of FTY-720 on distal TCR signaling is not due to inhibition of NFAT. The inhibitory effect of FTY-720 on T cells is mediated at least in part by PAK-1. Current studies are underway to further characterize the mechanism of FTY-720 mediated inhibition of distal TCR signaling. Together, these data provide novel insights into the effect of FTY-720 on human TCR signaling pathways.

398. Establishment of a New AAV Clinical Candidate for Prophylaxis Against Influenza A and B

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Influenza infections are the seventh leading cause of death in the U.S.A., equating to 49,000 deaths per year, which is a significant proportion of the almost 500,000 influenza-related deaths worldwide. Vaccines are not always effective at protecting humans from influenza. Furthermore, the emergence of a new influenza pandemic remains a threat that could result in substantial loss of life and worldwide economic disruption. The goal of our program was to develop an adeno-associated virus (AAV) vector-based prophylaxis regimen against influenza A and B as an alternative to the traditional influenza vaccine in the setting of seasonal and/or pandemic influenza infections. We first set out to discover novel AAV vectors with an enhanced performance profile in the airway epithelium. Using highly specialized molecular techniques designed to detect and isolate endogenous AAVs from a variety of human and non-human primate tissues, we isolated a novel AAV, AAVhu68, from human tissue. This vector exhibits a favorable phenotype that includes improved manufacturability and enhanced transduction across many different tissue types. Having selected AAVhu68 as the candidate AAV vector serotype, we focused our attention on optimizing the influenza antibody expression cassette. Unique to our influenza prophylaxis program is that the product consists of only one AAV vector that encodes a single open reading frame for the expression of a multi-domain antibody (MDAb). The AAVhu68.MDAb vector exhibited *in vivo* an impressive prophylaxis profile against lethal challenge with various influenza A and B strains. Specifically, when applied to the mouse nasal airway, the AAVhu68.MDAb vector conferred full protection against influenza A or B strains even when administered at very low doses (10^9 genome copies, GC). Interestingly, the protective low AAV vector dose resulted in the production of low level serum-circulating AAV-specific neutralizing antibodies. We have demonstrated that these neutralizing antibodies do not compromise effective vector readministration to the airway, which is beneficial should it be necessary to protect against a pandemic influenza strain that may arise during the window of effective universal AAV prophylaxis. The safety profile of the AAVhu68.MDAb vector is currently being evaluated in IND-enabling non-human primate studies in support of the progression of this AAV-mediated prophylaxis product into a phase I clinical trial.

399. Alpha-Defensin 5 Augments the Immuno-Modulatory Responses Elicited by an Adenovirus 26 Vaccine Delivery Vector in the Context of a Listeria Challenge Model

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Adenoviral vectors are widely utilized for vaccine delivery purposes, as they can elicit innate and humoral immune responses to an expressed antigen. Prior research suggests that alpha-defensins, effectors of the innate immune response, display inhibitory and augmentative properties in the course of a natural adenoviral infection. In the present study, we performed a direct comparison between adenovirus serotypes 5 (Ad5) and 26 (Ad26) and determined that these vectors have distinct phenotypes in response to pre-treatment with alpha-defensin 5 (HD5). While antigen expression by Ad5-HD5 is markedly inhibited both in vitro and in vivo compared to control infection, Ad26 has an opposing phenotype. We determined that HD5 pre-treatment enhanced the following properties of Ad26 compared to control infection: 1) Enhanced green fluorescent protein expression in vitro, 2) pro-inflammatory cytokines as examined by Luminex, 3) luciferase expression by IVIS imaging, and 4,5) serum-specific IgG and $CD8^+$ T cell levels in mice. The culmination of this data led us to believe that HD5 could potentially be utilized as an adjuvant in the context of Ad26 vaccine delivery. To test our theory, mice were immunized with low doses of an Ad26 vector expressing a protective antigen to Listeria. All animals treated with wild-type Ad26 vector (10^8) succumbed to Listeria infection, while 100% animals that were pre-treated with HD5 showed protection. These data demonstrate that HD5 can be utilized as an adjuvant as it positively modulates the immunogenicity of Ad26.

400. Bacteriophage Lambda as a DNA-VLP Vaccine Delivery Vector

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Background: Bacteriophage, bacterial viruses, can and have been exploited for decades for the application termed "phage display". This strategy involves the engineered expression of a peptide(s) of interest fused to the minor and/or major coat protein of a tolerant phage. Phage-based vaccines can function as valuable alternatives to naked DNA vaccination as the outer capsid can act to serve and protect DNA. Additionally, phage are natural immunostimulators acting as adjuvants that can stimulate both the humoral and cell-mediated immune responses. The viruses are exclusive to bacteria, meaning that they do not possess the capabilities to propagate within mammalian cells making them safe compared to their mammalian viral biased counterparts. **Methods and Results**: Bacteriophage λ has a proven track record of effective phage display fusion tolerance and in combination with a tuneable peptide display process the probability of fusion tolerance and functionality will be maximized. A specialized λ phage derivative was used to display various peptides to deliver the DNA cargo, including those that target professional antigen-presenting dendritic cells (DC). Our tuneable system involved the use of two genetic suppression systems in tandem to modulate cellular production of fused and unfused capsid alleles to maximize capsid viability, production, and fusion functionality. Manipulation of the phage genome was accomplished using simple genetic recombination strategies known as functional immunity assays, where the repression operon of the temperate phage is switched with the GOI by genetic selection for viable phage. Techniques and methods involving construction of the hybrid phage gene delivery vehicle involved simple and proven techniques in genetics, and molecular and phage biology. More specifically, a specialized HIV Gag-Env genetic construct was subcloned into a eukaryotic expression vector to ensure expression in the mammalian host, and the functional cistron was recombined into the phage. DNA encapsulated within the phage vector was assessed through DNase sensitivity assays where the complexes were incubated with DNaseI and the extent of DNA degradation was analyzed by gel electrophoresis. The stability of the vectors was assessed with respect to pH, temperature and time. The chimeric *gag-env* gene construct (courtesy and in collaboration with Dr. Chil-Yong Kang UWO) forms harmless, yet highly immunogenic self-assembling virus-like particles (VLP). DC targeted VLPs allow for the effective intracellular processing of viral immunogens and the stimulation of both the antiviral cell-mediated and humoral responses. The presence of *gag-env* genes in packaged phages was confirmed by PCR. The peptides \ evaluated included the tenth fibronectin fragment of the CD51 integrin that possesses an RGD peptide. This peptide is linked to rapid endocytosis and an induction of receptor-mediated endocytosis in DCs. The other peptide evaluated was the TAT peptide, a type of cell-penetrating peptide or CPP. In the future, DCs will be transfected with the phage vectors possessing the gag-env expression cassette. Cell targeting by the phage hybrid will be assessed using eGFP as an initial GOI and flow cytometry and intracellular VLP formation will be analyzed by electron microscopy. Transfection efficiency will be accessed using Western blot analysis on prepared lysates post-infection to look for a signal corresponding to the protein development of one or both of the desired protective antigens (Env and Gag).

401. Identification of a Novel Role of Src Kinases in Regulating T Cell Receptor (TCR) Independent T Cell Activation

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Introduction: T cell activation (TCA) through the T cell receptor (TCR) is the first step required to generate an effective T cell response. Engagement of the TCR with a peptide bound MHC complex present on the surface of antigen-presenting cells (APCs) initiates a series of intracellular signaling events culminating in the expression of pleotropic cytokines and signal transducing receptors. Inflammatory cytokines enhance TCA in a TCR-independent manner. Persistent TCA is detrimental and impairs T cell function. Thus, multiple mechanisms have evolved to regulate both TCR-dependent and independent signaling and maintain T cell homeostasis. Src-family kinases (SFKs) are required for activation of proximal TCR signaling pathways; however, the role of SFKs in TCR-independent TCA is unknown.

Here, we characterized the role of SFKs in TCR-independent TCA. **Methods**: Human T cell lines (Jurkat and HuT78) and primary human T cells obtained from healthy blood donors were treated with a selective inhibitor of SFKs (PP2). Following activation of distal TCR signaling with phorbol 12-myristate 13-acetate (PMA) and Ionomycin, TCA was measured by assessing IL-2 release (ELISA) and CD25 surface expression (flow cytometry). Activation and nuclear translocation of NFAT was assessed by immunoblots and NFAT-dependent promoter activity by luciferase assays. **Results:** Following TCR-independent TCA with PMA and Ionomycin, PP2 significantly increased IL-2 release and CD25 expression in both human T cell lines and primary human T cells. PP2 treatment did not alter expression of lymphocyte-specific Src-kinase (Lck) and nuclear factor of activated T cells (NFAT). However, inhibition of SFKs in human T cell lines and primary human T cells resulted in increased NFAT activation. This increase in NFAT activation resulted into its nuclear translocation, and enhanced NFAT-dependent promoter activity. Interestingly, the increase in NFAT activation following PP2 treatment was not due to increased intracellular calcium as PP2 treatment reduced calcium flux. **Conclusions:** Together, these data identified a novel role of SFKs in human T cells, and suggest that in addition to their role in initiating proximal TCR signaling, SFKs regulate TCR-independent TCA by preventing aberrant NFAT activation. This effect of SFKs likely contributes to maintain T cell homeostasis *in vivo* where T cells are constantly exposed to TCR-independent ligands such as inflammatory cytokines. Understanding mechanisms by which SFKs regulate both TCR-dependent and independent signaling may aid in the development of novel therapeutic strategies to reduce unwanted T cell response during inflammatory disease settings including gene therapies. **Acknowledgements**: This work was supported by the Intramural Research Program of the CBER, Food and Drug Administration. This project was supported in part by Dr. Alan Baer's appointment to the Research Participation Program at CBER administered by the Oak Ridge Institute for Science and Education through U.S. Department of Education and FDA.

402. Functional Assessment of Structural Reformatting and Protein Engineering Strategies for Therapeutic Gene Transfer Synthetic DNA-Plasmid Encoding Antibodies Against Ebola Virus Disease (EVD)

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Ebola virus disease (EVD) causes severe hemorrhagic fever in humans and is associated with high mortality rate. The Ebola epidemic of 2013-2015 in West Africa was by far the most fatal and the longest lasting in the recent history of emerging diseases. The challenges of such a large outbreak have underscored the need for effective EVD antiviral therapies and vaccines. While a vaccine for EVD was recently found effective in humans, vaccine protection is often not immediate and not beneficial in a therapeutic setting against acute cases of EVD . Passive immunization strategies that require the transfer of monoclonal antibodies (mAb) to confer immediate protective immunity have been used successfully in infectious disease settings including EVD. However, there are conceptual and methodological hurdles associated with antibody administration. This includes their production and optimization *in vitro*, their dosage *in vivo,* and finally the costs that are incurred during the manufacturing process. From this standpoint, the *in vivo* delivery of DNA-plasmid encoding antibodies offers an innovative, safe and cost effective approach to monoclonal antibody (mAb) administration. We have previously demonstrated *in vivo* electroporation (EP)-mediated gene delivery in mice of DNA encoding monoclonal antibodies (DMAbs) directed against various pathogens. The current study describes two DMAbs that target Ebola virus glycoprotein (EBOV-GP), EBOMAb-10 and EBOMAb-14, it also describes the functional impact of engineered modifications consisting of reformatting Immunoglobulin (Ig) to single chain antibody (scFv-Fc) and scaffold grafting within the aforementioned DMAbs structural frames. We report that EP-mediated gene transfer of EBOMAb-10 and EBOMAb-14 in their un-modified and modified formats leads to the secretion of functional antibodies in mice serum as assessed by EBOV-GP antigen binding by ELISA and viral neutralization in an EBOV-GP pseudotyped virus assay. We also report the disparate impacts that these structural changes have on EBOV-GP binding *in vitro* and in live cell expressing surface glycoprotein, as well as on viral neutralization. Taken together, the data described here provide the conceptual framework for the development of synthetic-DNA plasmid encoding monoclonal antibodies (DMAbs) with enhanced therapeutic potency against emerging diseases such as EVD. The study also provides the functional paradigm to assess the structural manipulations of DMAbs, and overall supports further animal testing *in vivo* to translate DNA-based passive immunization approaches into clinic in a safe and cost efficient manner

Liver-Based Therapy for Genetic and Metabolic Disease

403. Salmeterol, a Beta-Agonist, Enhances Therapeutic Efficacy in Pompe Gene Therapy

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Pompe disease results from acid alpha-glucosidase (GAA) deficiency, resulting in lysosomal glycogen accumulation and enlargement leading to progressive muscle weakness. Enzyme replacement therapy (ERT) with recombinant human (rh) GAA is currently the only FDA-approved treatment available in clinics. We developed gene therapy for Pompe disease using an adeno-associated viral (AAV) vector containing a liver-specific expression cassette to control GAA expression (AAV2/8-LSPhGAA), which could provide an alternative to ERT. We previously showed that adjunctive therapy with a β2-agonist, clenbuterol, improved therapeutic efficacy from gene therapy. Clenbuterol, a selective β2 receptor agonist, enhanced the receptor mediated uptake of

GAA through the cation-independent mannose-6-phosphate receptor (CI-MPR) by increasing CI-MPR expression and trafficking of GAA to lysosomes.

In this study we have evaluated an alternative β2-agonist, salmeterol, in GAA knockout (KO) mice and muscle specific CI-MPR/GAA "double" KO (mDK) mice during gene therapy. GAA activity was significantly increased in the heart, quadriceps, diaphragm, and gastrocnemius of two month old GAA-KO mice treated with vector (AAV2/8-LSPhGAA, $2x10^{11}$ vp/mouse) when compared with untreated GAA-KO mice. GAA activity was increased in the same muscles in vector-treated mDK mice that lacked CI-MPR in muscle, although to a lesser extent. These results showed the importance of CI-MPR for the uptake of GAA in cardiac and skeletal muscle. As expected, the glycogen accumulation was not efficiently reduced by vector and/or salmeterol administration in the skeletal muscles of mDK mice that lacked CI-MPR. In contrast, vector-treated GAA-KO mice that expressed CI-MPR had significantly reduced glycogen in skeletal muscles, including diaphragm ($p<0.01$), quadriceps ($p<0.01$), and gastrocnemius ($p<0.01$) following vector administration. Intriguingly, salmeterol (30 mg/L in drinking water) alone reduced glycogen content in skeletal muscles, including diaphragm ($p< 0.01$), quadriceps ($p<0.01$), and gastrocnemius ($p<0.05$). However, this reduction from salmeterol alone was not observed in heart. In addition, glycogen content was reduced to a greater extent by the combination of vector and salmeterol administration in the quadriceps ($p<0.05$) and gastrocnemius ($p<0.05$) in comparison with vector alone, but this synergistic effect was not observed in heart. Autophagosome accumulations, a secondary effect of Pompe disease, were reduced in skeletal muscle by vector administration. In addition, salmeterol increased latency significantly in the wirehang test ($p<0.05$), and improved growth as demonstrated by increased body weight ($p<0.05$). Salmeterol induced muscle hypertrophy as demonstrated by increased gastrocnemius weight, in combination with vector ($p<0.01$). In contrast, vector administration alone reduced cardiac hypertrophy from Pompe disease, but it did not induce skeletal muscle hypertrophy. Thus, further development of salmeterol as adjunctive therapy is justified as a method for enhancing the therapeutic effect of GAA replacement with gene therapy or ERT for Pompe disease.

404. Anc80 Mediates Hepatic Correction of Methylmalonyl-CoA Mutase Deficiency in Murine Models of Methymalonic Acidemia

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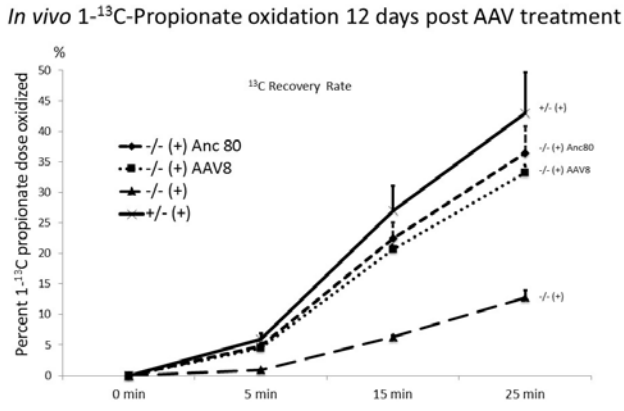
Other than dietary and cofactor therapy, no alternative to organ transplantation exists for patients with isolated methylmalonic acidemia (MMA), a common and severe organic acidemia most frequently caused by mutations in the enzyme methylmalonyl-CoA mutase (MUT). *Mut* knock-out ($Mut^{-/-}$) mice replicate 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 systemic 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 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 received $5x10^{12}$ GC/kg of either Anc80 or AAV8 vector (n=3 per group) delivered by retro-orbital injection. Plasma methylmalonic acid and methylcitrate concentrations and weight were measured before and post AAV gene therapy on day 12, 30 and 60. 1-^{13}C propionate oxidative capacity was measured on day 12 post AAV gene therapy. Both vectors induced a robust biochemical and clinical response by day 12. Plasma methylmalonic acid levels dropped from 985±86 uM to 173±28 uM for the Anc80 vector and from 1153±511 uM to 176±31 uM for the AAV8 vector, and were paralleled by substantial weight gain from 20.1±1.9g to 26.2±2.5g for the Anc80 vector and from 21.9±2.9 g to 24.3±2.8g for the AAV8 vector. A significant increase in the oxidative capacity for propionate (see figure) were observed on D12 post Anc80 or AAV8 gene therapy. The AAV treated animals maintained their weight and metabolic stability on D30 and 60, but showed no significant changes compared to the D12 time point. In addition, Anc80-hAAT-MUT vector was able to rescue the lethal phenotype in the neonatal $Mut^{-/-}$ mice model at dose as low as $1x10^{10}$ GC/pup. These studies show the functional equivalency of AAV8 and Anc80 vectors for the correction of hepatic *Mut* deficiency in mouse models of MMA, and demonstrate the utility of the $Mut^{-/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$ mice to rapidly assay vector efficacy. The findings support further investigation of Anc80-hAAT-MUT with the goal to develop an effective gene therapy that can be effective in patients with pre-existing antibodies to naturally occurring AAV serotypes.



405. Engineered AAV Capsids with Combined High Human Liver Transduction In Vivo and Unique Humoral Seroreactivity

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Existing recombinant adeno-associated virus (rAAV) serotypes for delivering *in vivo* gene therapy treatments for human liver diseases have not yielded combined high-level human hepatocyte transduction and favorable humoral neutralization properties. Yet, these combined properties are important for therapeutic efficacy. To bioengineer capsids that exhibit both unique seroreactivity profiles and functionally transduce human hepatocytes at therapeutically relevant levels, we performed multiplexed sequential directed evolution screens using diverse capsid libraries in both primary human hepatocytes *in vivo* and with pooled human immunoglobulins. AAV libraries were subjected to five rounds of *in vivo* selection in mice with humanized livers to isolate an enriched human-hepatotropic library that was then used as input for a sequential on-bead screen against pooled human immunoglobulins. Evolved variants were vectorized and validated against existing hepatotropic serotypes under clinical consideration. Two of the evolved rAAV serotypes—NP40 and NP59—exhibited both superior functional human hepatocyte transduction *in vivo* in chimeric humanized liver mice, along with favorable human seroreactivity profiles. This demonstrates the importance of combining key parameters such as utilizing replicating AAV libraries that allow for selection beyond just hepatocyte receptor binding and entry, evolving human hepatocyte tropism in human rather than mouse hepatocytes *in vivo*, screening for humoral evasion against pools of human immunoglobulins from thousands of patients, and assessing transduction using clinically meaningful methodologies. The result is a panel of novel rAAV variants with superior human hepatic transduction and unique humoral neutralization compared to previously characterized serotypes. These novel capsids represent enhanced vector delivery systems for future human liver gene therapy applications.

406. Ancestral AAV Viruses as a New Platform for the Treatment of Liver Inherited Diseases: Wilson Disease

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Wilson's disease (WD) is an autosomal recessively inherited copper storage disorder due to mutations in the ATP7B gene that causes hepatic and neurologic symptoms. Current treatments are based on lifelong copper chelating drugs, which may cause side effects and do not restore normal copper metabolism. We have recently demonstrated that the administration of an AAV8 vector expressing ATP7B or truncated variants of the protein into a WD animal model resulted in a dose-dependent therapeutic effect. In the present study we have compared the therapeutic efficacy of the AAV8 vector and the Anc80 vector, a novel serologically unique synthetic AAV-like particle. Both vectors carried one of the truncated version of the ATP7B (named ATP7B-T2). WD animals were treated with the same dose of each vector. Therapeutic efficacy was equivalent between the 2 vector systems, as manifested by a profound reduction of serum transaminases and urinary copper excretion and normalization of serum holoceruloplasmin. The livers of treated AAV8-ATP7BT2 or Anc80-ATP7BT2 animals showed normalization of copper content and absence of histological alterations. Our data demonstrate that Anc80-ATP7BT2 mediated gene therapy provides long-term correction of copper metabolism in a clinically relevant animal model of WD and that Anc80 might represent a promising platform for the development of gene therapy for inherited liver diseases, providing support for future translational studies.

407. Safety and Efficacy of an AAV-Based Liver Gene Therapy for Crigler-Najjar Syndrome

Fanny Collaud[1], Giuseppe Ronzitti[1], Giulia Bortolussi[2], Laurence Guianvarc'h[1], Philippe Veron[1], Séverine Charles[1], Patrice Vidal[1,3], Marcelo Simon Sola[1,3], Christian Leborgne[1], Stephanie Rundwasser[1], Ludivine Dejoint[1], Delphine Goncalves[1], Laetitia Van Wittenberghe[1], Bernard Gjata[1], Samia Martin[1], Christine Le Bec[1], Piter J. Bosma[4], Andres F. Muro[2], Matthias Hebben[1], Federico Mingozzi[1,3,5]

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Crigler-Najjar (CN) syndrome is a rare and life-threatening monogenic disease caused by mutations in the uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) gene. The liver-specific enzyme UGT1A1 catalyzes the glucuronidation and excretion of unconjugated bilirubin (UCB). Accumulation of neurotoxic UCB in

the body caused by the reduction or absence of UGT1A1 activity can lead to severe and irreversible neurological damage and ultimately death. For patients suffering from CN syndrome, current therapeutic options are limited, including intensive phototherapy (up to >12 hours per day) and liver transplantation. Gene therapy represents a real hope to provide these patients with a safe and effective curative treatment. Adeno associated viral (AAV) vectors are the tool of choice for in vivo gene transfer. Encouraged by remarkable results in the clinic with AAV vectors and with the aim of treating CN syndrome, we developed an optimized rAAV vector carrying human UGT1A1 sequence under the control of a liver-specific promoter. After one single administration at a clinically-relevant dose, we demonstrated durable correction of the disease phenotype in two animal models of the disease. Thanks to an efficient manufacturing process based on the triple-transfection of HEK293 cells grown in suspension, we generated large-scale high-quality vector preparations with characteristics identical to those of small scale produced in adherent cells. Pre-clinical studies and a GLP toxicology/biodistribution study were also conducted, demonstrating the safety and efficacy of the rAAV-UGT1A1 vector. Studies conducted included the evaluation of corticosteroid administration in treated animals, a measure that may be necessary in the clinic to modulate vector-related immunogenicity. In conclusion, this work lays the fundaments for a liver-directed gene therapy trial for Crigler-Najjar syndrome set to start in the near future.

408. AAV Vector-Based Gene Therapy Strategies for Type 3 Glycogen Storage Disease

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Glycogen storage disease type III (GSDIII) is a recessive disorder due to mutations in the glycogen debranching enzyme (GDE), one of the two enzymes involved in the cytosolic glycogen degradation. The lack of GDE leads to glycogen accumulation in all tissues, especially in muscle. During childhood, GSDIII is mainly a metabolic disease, being characterized by hepatomegaly and fasting hypoglycemia. During this phase the disease is managed by uncooked cornstarch supplied in a strict dietary regimen. In the adulthood, the metabolic manifestations tend to disappear and muscle weakness becomes prominent. At present, no curative treatment is available for GSDIII. Here, we derived a new KO mouse model of GSDIII (GDE-/-). GDE-/- mice lacked GDE expression and accumulated glycogen in virtually all tissues. Glycogen accumulation resulted in hypoglycemia and muscle weakness. One of the limitation in the development of a gene therapy for GSDIII based on adeno-associated virus (AAV) vectors is their limited packaging capacity (~5 Kb). Being the coding sequence of GDE 4.6 Kb, this transgene hardly fits into a single AAV vector. To overcome this limitation, and based on promising in vitro results (Sun B. 2013), we first tested overexpression of the lysosomal enzyme acid alpha-glucosidase as a therapy for GSDIII. However, this approach resulted only in correction of liver glycogen accumulation. Next, we tested dual-AAV vectors to restore GDE activity body wide. Dual AAV vectors expressing GDE were intravenously injected in GDE-/- mice. This resulted in rescue of the disease phenotype. In conclusion, our work represents the first proof-of-concept study of successful gene therapy in the mouse model of GSDIII.

409. *Ex Vivo* Gene Therapy in a Pig Model of Hereditary Tyrosinemia Type 1: Comparing Single Cell vs. Spheroid Suspension Hepatocytes for Intraportal Infusion

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Purpose: To treat and cure a pig model of hereditary tyrosinemia type 1 (HT1) through *ex vivo* gene delivery via intraportal transplantation of single cell or spheroid suspension hepatocytes, and compare the two methods in terms of safety and efficacy. Methods: We performed laparoscopic partial hepatectomies on six-week-old (15-20 kg) fumarylacetoacetate hydrolase (*FAH*)-deficient pigs (n=6) and isolated primary hepatocytes *ex vivo*. Hepatocytes were transduced in suspension at multiplicity of infection (MOI) 20 TU with lentiviral vectors containing the porcine *FAH* and the sodium-iodide simporter (*NIS*) cDNA under control of the liver-specific, human thyroxine-binding globulin (TBG) promoter. The NIS reporter is a non-invasive method to monitor hepatocyte expansion using positron emission tomography-computed tomography (PET-CT). This procedure was repeated in two wild-type pigs, and their hepatocytes were labeled with Zirconium-89 to evaluate cell biodistribution also through PET-CT. All animals received autologous hepatocyte transplantation by percutaneous portal vein infusion of single cell or spheroid suspension. Portal pressures were measured during transplantation and ultrasound used to evaluate the presence of thrombotic events. Engraftment and expansion of *ex vivo* corrected autologous hepatocytes were followed through biochemical and histological analysis, PET-CT imaging, and through the animal's ability to thrive off the protective drug 2-(2-nitro-4-trifluoromethylbenzyol)-1,3 cyclohexanedione (NTBC). Results: Animals receiving single cell suspension hepatocytes, 4.8-15.0g, experienced a mean change in portal pressure of 0.8-6.0mmHg during injection. No thrombus was noted. Animals receiving spheroid suspension hepatocytes, 9.1-10.8g, experienced a mean portal pressure change of 10.9-12.5mmHg. Portal vein thrombi were noted in two animals and portal infusions were stopped. These animals were treated

with therapeutic enoxaparin for seven days at which time ultrasounds showed no evidence of thrombus. On PET-CT imaging immediately, 24 hours, and 72 hours post-operatively, and on radioactivity analysis 72 hours post-operatively, no significant difference in biodistribution was found between single cell and spheroid hepatocytes. Animals transplanted through both methods are healthy and currently being cycled on and off NTBC to stimulate expansion of corrected hepatocytes. Conclusions: In this pre-clinical study we show that *ex vivo* gene correction of autologous hepatocytes in FAH-deficient pigs can be performed using single cell or spheroid suspension hepatocytes, with single cell suspension allowing for infusion of larger numbers of hepatocytes. In addition, transplantation of larger spheroids presents a higher short-term risk for portal vein thrombosis and increased portal pressures.

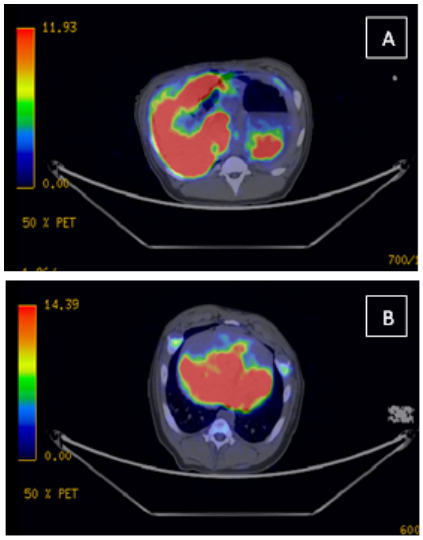

Figure 1. PET-CT imaging of Zirconium-89-labeled single cell (A) vs. spheroid (B) suspension hepatocytes shows no significant difference in biodistribution.

410. Liver Transplantation in Methylmalonic Acidemia as a Model for Developing Outcome Parameters for a Gene Therapy Clinical Trial

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Isolated methylmalonic acidemia (MMA), caused by a defect in methylmalonyl-CoA mutase (MUT), is a severe inborn error of metabolism associated with high morbidity and mortality. Liver (LT) or combined liver and kidney (LKT) transplantation have been increasingly used in the most severely affected patients. Although functional MUT enzyme in the transplanted hepatic tissue can prevent recurrent episodes of metabolic decompensation, this approach comes with surgical complications and the need for lifelong immunosuppression, while patients remain at risk for extrahepatic complications. The experience from longitudinal studies in liver transplant recipients will guide the development of outcome parameters for alternative and less invasive therapeutic approaches, such as gene therapy. We therefore investigated long term clinical and biochemical outcomes in 16 patients with *mut* MMA (11 LKT, 5 LT; ages 3-38y), including 10 patients studied before and after the LT/LKT procedures. Patients were followed up to 18.2 y post-transplant at the NIH Clinical Center as part of a dedicated natural history study. Longitudinal growth measurements (mean follow up time 6y) among pediatric patients (N=11; 8 males, 3 females) showed no statistically significant changes overall in height-for-age *z*-scores post-transplant, although *z*-scores improved by 1-1.59 in 4/11 patients. In the LT recipients, estimated glomerular filtration rate (eGFR) values remained stable up to 5 (65.5 ± 5.2 ml/min/1.73m^2; N=3) and 15 years post-transplant (83.1 ± 52.6 ml/min/1.73m^2; N=2), provided patients remained on protein restriction, suggesting LT may stabilize renal dysfunction. Plasma methylmalonic acid concentrations decreased from 3223 ± 2675 μM, (nl < 0.4 μM; N=11) prior to transplant to 179 ± 94 μM ($p < 0.05$; N=6) within 1 year after transplant, 276 ± 176 μM ($p < 0.01$; N=10) within 1-5 years, 361 ± 267 μM ($p < 0.05$; N=5) within 5-10 years, and 569 ± 612 μM ($p < 0.05$; N=5) over 10 years post-transplant.

We previously used gene therapy-treated and transgenic MMA mice to develop 1-^{13}C-propionate oxidation breath testing as a more reliable biomarker of restored *in vivo* MUT activity. Translating this method to humans, 1-^{13}C-propionate was administered as a single oral bolus, and breath samples were collected serially over 2 hours to measure $^{13}CO_2$ enrichment. Propionate oxidation was lower in all non-transplanted *mut* patients (N=20) compared with controls (N=16; $p < 0.0001$), while both LT and LKT recipients demonstrated oxidation rates at control levels (N=8; p = NS). Notably, a 329% and 1063% increase in 1-^{13}C-propionate oxidation was observed in two *mut* patients tested before and after transplant (1 LKT, 1 LT). These improvements were detected despite plasma methylmalonic acid levels remaining massively elevated (121 and 402 μM, respectively). Biomarkers discovered in gene therapy treated and transgenic mice are currently being evaluated in transplant patients to identify additional laboratory endpoints for future human gene therapy trials.

411. Liver Transduction Efficacy with AAV Vectors in Juvenile *UGT1A1*-Deficient Mice

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Crigler-Najjar syndrome (CN) is an ultra-rare monogenic liver disease. Severely affected patients have high levels of serum unconjugated bilirubin, which can lead to brain damage and death. Treatment of CN is based on phototherapy for 10-12 hours per day to convert unconjugated bilirubin into soluble photoisomers without the need of conjugation. This practice is inconvenient and its efficacy decreases with age. Indeed, liver transplantation is the only curative option for these patients. Thus, CN syndrome is a disease with high unmet medical need. Aiming to provide an effective and long-lasting therapy for CN,

we have previously developed an AAV8 vector optimized for the liver expression of the UGT1A1 transgene (AAV8-hUGT1A1), showing safe and effective correction of total serum bilirubin (TB) levels, both in mouse and rat models of the disease. However, due to the non-integrative nature of AAV vectors, vector dilution in actively dividing hepatocytes is a major obstacle to sustained long-term transgene expression following AAV-mediated gene therapy in pediatric/juvenile subjects. Here, we determined vector efficacy in neonatal and juvenile Ugt1$^{-/-}$ mice to model gene transfer in pediatric subjects and defined the age and vector dose parameters for safe and long-lasting correction of the genetic defect.To this end, the AAV vector was administered at different developmental ages 4, 11, and 18 days after birth (P4, P11, and P18, respectively) and dose finding studies were performed in the short-term. Total bilirubin (TB) levels measured at one month of age showed dose-dependent partial correction of the disease phenotype in mice treated at P4. At P11, vector transduction of the liver was superior, with complete elimination of TB from serum at the highest vector dose tested (5E12 vg/kg). According to this trend, in mice injected at P18 the dose that resulted in complete correction of TB accumulation was one log lower, 5E11 vg/kg. Long-term studies were performed for this time point and therapeutic efficacy was evaluated in a long-term follow-up study (up to 6 months of age). No adverse effects were observed in the animals injected at the highest doses. Additionally, the developmental state of the liver at the time of injection was analysed by different markers of proliferation and correlated with AAV vector persistency. In conclusion, our data demonstrate the safety and efficacy of juvenile gene transfer for Crigler-Najjar syndrome in a relevant animal model of the disease and provide tools and a strong rationale for the translation of these results to humans.

412. Sustained Inhibition of Hepatitis B Virus Replication with AAV Vectors Expressing Primary micro RNA Mimics

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Despite the availability of a vaccine against hepatitis B virus (HBV) infection, chronic HBV infection still remains a global problem. This is worsened by the fact that current drugs are poorly effective. This has resulted in growing efforts to develop a more potent therapy for the chronic carriers of the virus. Activation of the RNA interference (RNAi) pathway with exogenously supplied mimics of RNAi pathway intermediates to counter HBV infection has been extensively studied. Mimics of primary microRNAs (pri-miRs) showed superior features over synthetic small interfering RNAs or expressed mimics of precursor microRNAs. These include the fact that pri-miRs are compatible with pol II promoters, which allows tissue specific expression. However, lack of effective and safer delivery system for anti-HBV pri-miRs hampers their progress into advanced stages of clinical trials. This study assessed the feasibility of using recombinant adeno-associated viruses (AAVs) as a delivery vehicle for anti-HBV pri-miRs. AAVs expressing mimics of human pri-miR-31 resulted in up to 8 months pri-miR expression and significant inhibition of HBV replication in transgenic mice. These encouraging observations were accompanied by lack of liver toxicity or induction of inflammatory response. This suggests that AAVs expressing anti-HBV pri-miR-31 mimics can be a safe and effective alternative treatment for chronic HBV infection.

413. Correction of Ornithine Transcarbamylase Deficiency Following Treatment with PhaseRx's Hybrid mRNA TechnologyTM Delivery System and Safety Evaluation in Rats and Non-Human Primates

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We have developed a two-component platform, comprising a polymer that promotes endosomal escape plus an inert mRNA lipid nanoparticle for efficient in vivo delivery of therapeutic mRNA to the liver. The Hybrid mRNA Technology™ platform allows development of intracellular enzyme replacement therapy (i-ERT) which targets the synthesis of delivered mRNA to hepatocytes to treat genetic diseases lacking specific enzymes solely or predominantly expressed by hepatocytes. We have used our optimized formulation containing a therapeutic mRNA encoding Ornithine Transcarbamylase (OTC) protein to treat a mouse disease model recapitulating OTC deficiency (OTCD), a urea cycle disorder. Urea cycle disorders result from single gene mutations that lead to deficiency in one of the six enzymes in the urea cycle pathway. This deficiency can trigger hyperammonemia, a life-threatening illness that leads to brain damage, coma or even death in humans. The deficient protein is mitochondrial so IV protein therapeutics are ineffective due to the challenge in transporting large molecules across the plasma membrane. Liver transplantation is the only cure for urea cycle disorders but is limited by the shortage of donors and complications associated with rejection and infection of the transplant. There is a dire need for new treatment options. With our OTC mRNA formulated with the Hybrid mRNA Technology system, we have successfully demonstrated correction of a hyperammonemia OTCD mouse model. Treatment with therapeutic OTC mRNA resulted in prolonged survival, normalization of blood ammonia and urinary orotic acid. OTC protein expression is detected in the liver after a single mRNA dose with long durability. The treatment was well tolerated, with no toxicities associated with both single and multiple dosing regimens. We have also shown enhanced hematopoiesis in naïve rats and non-human primates when dosed with erythropoietin (EPO) mRNA. No LFT or cytokine changes were observed in the large animal studies. PhaseRx's Hybrid mRNA Technology™ platform provides a significant opportunity for the development of potent and safe therapeutic mRNA-based i-ERT for urea cycle disorders and other orphan liver diseases.

414. High and Prolonged Sulfamidase Secretion by the Liver of MPSIIIA Mice Following Hydrodynamic Tail Vein Delivery of Antibiotic-Free pFAR4 Plasmid Vector

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Mucopolysaccharidosis type IIIA (MPS-IIIA) or Sanfilippo A syndrome is a lysosomal storage genetic disease that results from the deficiency of the N-sulfoglucosamine sulfohydrolase (SGSH) protein, a sulfamidase required for the degradation of heparin sulfate glycosaminoglycans (GAGs). The accumulation of these macromolecules leads to somatic organ pathologies, severe neurodegeneration and death. To assess a novel gene therapy approach based on prolonged secretion of the missing enzyme by the liver, mediated by hydrodynamic gene delivery, we first compared a kanamycin and an antibiotic-free expression plasmid vector, called pFAR4. Thanks to the reduced vector size, pFAR4 derivatives containing either a ubiquitous or a liver-specific promoter mediated a higher reporter gene expression level than the control plasmid. Hydrodynamic delivery of SGSH-encoding pFAR4 into MPS-IIIA diseased mice led to high serum levels of sulfamidase protein that was efficiently taken up by neighboring organs, as shown by the correction of GAG accumulation. A similar reduction in GAG content was also observed in the brain, at early stages of the disease. Thus, this study contributes to the effort towards the development of novel biosafe non-viral gene vectors for therapeutic protein expression in the liver, and represents a first step towards an alternative gene therapy approach for the MPS-IIIA disease.

Musculo-skeletal Diseases II

415. AAV-Mediated Gene Therapy for Rheumatoid Arthritis: Phase Ib First-in-Human Study with ART-I02

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Rheumatoid arthritis (RA) is a systemic, chronic, inflammatory disorder leading to pain, swelling and limited motion of joints, ultimately resulting in destruction of articular cartilage and bone, joint deformities and disability in the majority of patients. RA is the most common type of autoimmune arthritis, affecting approximately 1% of the population (1.3 million people) in the US. Despite current treatment options, up to 50% of RA patients are suffering from one or more joints displaying persistent signs of inflammation. Arthrogen is taking a gene therapy approach to improve treatment of these mono- and oligoarthritis patients. The gene therapy that is developed is ART-I02, an adeno-associated viral vector (AAV) of serotype 2 pseudotyped with serotype 5 capsids encoding the human transgene interferon-β (hIFN-β). Transcription of the hIFN-β mRNA is controlled by an inflammation-inducible nuclear factor κB (NF-κB) responsive promoter. Thus, hIFN-β will be expressed in inflamed joints in RA patients, while expression of hIFN-β in other (non-inflamed) tissue throughout the body is minimized, providing a potentially stronger safety profile. A single dose of ART-I02 is delivered in the joint, a site of endogenous hIFN-β expression. Proof of principle was obtained in two models of arthritis: adjuvant arthritis in rats and collagen induced arthritis in non-human primates. Injection of ART-I02 or the corresponding vector expressing rat IFN-β in the joints of these animals resulted in expression of IFN-β and reduced swelling and inflammation. Furthermore, decreased bone erosion was observed in the injected joint. Toxicology and biodistribution studies in rats further confirmed the good safety profile of the ART-I02 vector. Altogether, these efficacy and safety studies form the basis for the execution of a first-in-human study with ART-I02. This is a phase Ib open label dose escalating study to investigate the safety of a single intra-articular ART-I02 injection in RA patients with active arthritis in a joint. Subjects are followed for 24 weeks for safety and signs of effect and are subsequently rolled over in a long term follow-up study for another 4.5 years to assess long term safety. The design of this study and the path to regulatory approval and enrollment of RA patients will be presented.

416. Single Systemic AAV Micro-Dystrophin Therapy Ameliorates Muscular Dystrophy in Young Adult Duchenne Muscular Dystrophy Dogs for Up to Two Years

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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. The 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 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 the dose of 0.5 (N=1, low), 1 (N=2, medium) and 3 (N=2, high) $x10^{14}$ viral genome particles/kg. All dogs received transient immune suppression and tolerated the infusion with no adverse events. Blood panels were unremarkable for up to two years. Muscle biopsy revealed sustained micro-dystrophin expression to the last biopsy time

point (20 months for the low and medium doses and 6 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. Limb muscle force was significantly enhanced. 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. Similar therapy in human patients may greatly improve the disease course (Supported by NIH, DOD, Jesse's Journey and Solid GT).

417. A Blinded, Placebo-Controlled Systemic Gene Therapy Efficacy Study in the GRMD Model of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a progressive and body-wide muscle wasting disease that is caused by the absence of the membrane-stabilizing protein, dystrophin. While mouse models of DMD may provide insight into the potential of a corrective therapy, studies in the dystrophin-deficient golden retriever muscular dystrophy (GRMD) model may provide more readily translatable information related to biodistribution, efficacy, durability and safety. To evaluate the clinical translatability of a novel adeno-associated virus-9 (AAV-9)-microdystrophin (SGT-001), GRMD dogs were systemically treated at 3 months of age in a blinded, single ascending dose study and followed for 91 days post-dosing.

12 GRMD dogs were screened for pre-existing immunity to both AAV8 and AAV9 and subsequently phenotypically stratified into one of four cohorts (n=3/cohort). Animals were administered a single intravenous dose of either: manufacturing excipient (placebo), $1x10^{13}$ vg/kg, $1x10^{14}$ vg/kg or $2x10^{14}$ vg/kg of canine SGT-001. All animals received transient immunosuppression with 1 mg/kg prednisone for a total of 5 weeks from D-7 through D+28 . A comprehensive suite of analyses were performed that included biodistribution, protein expression, histopathology, clinical pathology and assessments of immune response. Functional parameters that were investigated included limb muscle force, MRI, respiratory function and cardiac function. Dose-dependent increases in protein expression were concomitantly observed with improvements in histopathology. Similarly, vector genome copy number exhibited a dose-dependent increase in both muscle and non-muscle tissues. All administrations were well tolerated. These data, in combination with statistically significant observed functional benefits, suggest that systemically administered SGT-001 may be administered safely and is a potentially disease correcting therapy for DMD.

418. Application of a CRISPR/Cas9 Gene Editing Therapy in a Novel Humanized Dystrophic Mouse Model of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a progressive, degenerative muscle disease, typically caused by out-of-frame mutations in the *DMD* gene. Potential therapeutic strategies, such as exon skipping or clustered, regularly interspaced, short palindromic repeats (CRISPR) and -associated protein (Cas) 9, aim to restore the *DMD* reading frame to turn Duchenne into the milder, allelic disease, Becker muscular dystrophy. Currently, there are no dystrophic mouse models that allow for testing of such therapies in the context of the human *DMD* sequence *in vivo*. Therefore, we have created a novel mouse model whereby we have used the hDMD mouse (containing the full human *DMD* gene integrated into chromosome 5) and mutated it to be out-of-frame by deleting exon 45 using CRISPR/Cas9 in hDMD zygotes. We have crossed these hDMD del45 mice to mdx and mdx D2 backgrounds, both of which lack mouse dystrophin, to create a fully dystrophic model containing the human *DMD* gene. We have utilized this model to demonstrate proof of principle that our CRISPR/Cas9 platform, which targets deletion of human *DMD* exons 45-55, can be directly applied *in vivo* to restore dystrophin expression. These studies demonstrate that the hDMD del45 mdxD2 mouse is a useful model for pre-clinical testing of genetic therapies that act on the *DMD* gene and provide proof of principle that our CRISPR based gene editing strategy is functional *in vivo*.

419. Efficacious Androgen Hormone Administration in Combination with AAV Vector-Mediated Gene Therapy in Female Mice with Pompe Disease

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Pompe disease is a lysosomal storage disorder caused by deficiency of acid α-glucosidase (GAA), and makes weakness in many organs and tissues including cardiac and skeletal muscles. Multiple studies have

confirmed that the sex-dependent decrease in the efficacy of adeno-associated virus (AAV) vectors in female mice also applies to Pompe disease, including our pharmacology-toxicology study of an AAV2/8 vector containing a liver-specific promoter to drive expression of GAA (AAV2/8-LSPhGAA). GAA activity was significantly higher and glycogen content was lower in the liver, heart, and quadriceps of male mice following vector administration, in comparison with female mice. Androgens such as testosterone are expected to enhance the efficacy of an AAV vector in female mice with Pompe disease by multiple effects, including increased transduction from the AAV vector and muscle hypertrophy. We evaluated the effect of testosterone in female mice GAA knockout (KO) mice. Mice were treated with testosterone prior to AAV2/8-LSPhGAA vector administration. Female and male GAA-KO mice (8 of each sex) were be implanted subcutaneously with pellets containing testosterone propionate prior to of AAV2/8-LSPhGAA administration. Control vector-treated GAA-KO mice (n=8 of each sex) were implanted sham pellets. Groups of GAA-KO mice (n=8 of each sex) were injected with PBS as a negative control group for this experiment, and implanted with either testosterone or sham pellets. Groups of GAA-KO mice were euthanized for tissue analysis at 6 weeks following testosterone (or sham) treatment and vector administration. Neuromuscular function improved as demonstrated by increased rotarod latency for female mice treated with testosterone and vector, in comparison with vector alone ($p<0.05$). Muscle strength likewise increased as demonstrated by increased wirehang latency for female mice treated with testosterone and vector ($p<0.01$). Biochemical correction improved following the addition of testosterone as demonstrated by increased GAA activity in the heart, diaphragm, quadriceps, extensor digitorum longus, and soleus muscles for female mice treated with testosterone and vector, in comparison with vector alone ($p<0.01$). Furthermore, glycogen content was reduced by the addition of testosterone in the diaphragm ($p<0.01$). These data confirmed the benefits of brief treatment with an androgen hormone in mice with Pompe disease, and support the further development of related drugs in order to increase the efficacy from AAV vectors.

420. AAV.ANO5 Rescue of Muscle Dysfunction in *ANO5* Mutant Mice Provides a Pathway Toward Treatment of LGMD2L

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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, depending upon patient population, with estimates of 1:50,000 individuals. There is no treatment or cure for LGMD2L. Previously, we generated an *Ano5* mutant mouse using a gene trap method. *Ano5* mutant mice were viable and recapitulated LGMD2L phenotypes including: reduced force generation in the diaphragm, defects in membrane repair ability, impaired regeneration following acute injury, and aberrant mitochondrial function. *In vitro* analysis of *Ano5* mutant-derived myoblast cultures confirmed deficiencies in membrane repair following injury and myogenic fusion events. In this study, we have utilized these *Ano5* mutant mouse phenotypes to assess the efficacy of gene replacement via intramuscular delivery of an AAV.ANO5 vector. Experiments are ongoing to assess AAV.ANO5 rescue of regeneration defects following injury, as well as promotion of myotube fusion *in vitro*. We successfully delivered AAV.ANO5 and verified expression of hANO5 following intramuscular injection into the tibialis anterior (TA) muscle. In the TA of *Ano5* mutant males, which have reduced activity of the mitochondrial enzyme citrate synthase (CS), AAV.ANO5 treatment increased CS activity. We also demonstrated that AAV.ANO5 treatment improved the ability of flexor digitorum brevis (FDB) fibers to reseal following laser injury. These data provide the first evidence that an AAV.ANO5 therapy can restore normal muscle functions to *Ano5*-deficient tissues; they lay a path for systemic delivery studies in animal models, and ultimately progression toward a clinical therapy for LGMD2L patients.

421. Antisense Targeting of Dynamin 2 by Intramuscular Delivery of Vivo-Morpholinos Rescues the Pathology in Myotubular Myopathy Mice

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Centronuclear myopathies (CNM) are a group of inherited congenital disorders characterized by muscle weakness and common histopathological features, such as central nuclei in myofibers. They are caused by mutations in at least 5 genes, *MTM1*, *DNM2*, *BIN1*, *RYR1* and *TTN*, some of which may be involved in common pathophysiological pathways. The most severe and frequent form, X-linked myotubular myopathy (XLMTM), results from loss-of-function mutations in the gene encoding myotubularin (MTM1), with subsequent increased levels of dynamin 2 (DNM2) in skeletal muscles. In the present study, we developed a therapeutic approach aimed at reducing dynamin 2 levels during the postnatal period by using specific antisense vivo-morpholinos. We screened various *Dnm2* morpholino oligomers *ex vivo* and selected the two most efficient for *in vivo* studies. Intramuscular administration of these vivo-morpholinos in myotubularin-deficient mice normalized dynamin 2 protein levels and ameliorated muscle fiber size and organelle distribution. Most importantly, the contractile force of targeted muscles also increased upon treatment. These results indicate that dynamin 2 knock-down by antisense oligonucleotide-based compounds represents a novel therapeutic strategy for myotubular myopathy.

422. Improved Quality of Life in Dogs with OA After Intra-Articular XT-150 hIL-10v Plasmid DNA

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Osteoarthritis (OA) is a disease of unknown etiology that manifests as significant impairment in joint function resulting from degeneration and destruction of tissues within the synovial capsule. Over 25 million sufferers in the US, and millions more world-wide, are poorly treated by current therapeutic options that target symptomatic relief. Importantly, this disease is also prevalent in the veterinary population and is especially common in dogs, cats, and horses. An important component of OA in all species is the production of proinflammatory cytokines within the joint capsule and the resultant alteration of cell functions within the joint. Interleukin-10 (IL-10) can reverse proinflammatory cytokine effects, but must be provided long-term for efficacy. IL-10-encoding plasmid-based gene therapies can provide long-term efficacy in models of neuropathic pain, another chronic condition driven by derangements in proinflammatory cytokine function. Here we describe the results of a pilot double-blind placebo-controlled study examining an aqueous formulation of this plasmid-based therapy, XT-150, using companion dogs with OA as a disease model. We demonstrate that XT-150, which encodes a proprietary variant of the human IL-10 gene, delivered intra-articularly in the dog is well-tolerated and can provide long-term improvements in overall quality of life and joint function in these animals. In addition, animals that had originally been in the placebo group that were then administered XT-150 showed remarkable behavioral improvements. Because of the similar natural history and progression of OA in human and veterinary populations, these data are supportive of translation of IL-10-based therapies into the clinic in both populations.

423. Safe and Effective Cell Therapy Using Dental Pulp Stromal Cells for Duchenne Muscular Dystrophy

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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 stromal cells (DPCs) could be potential therapeutics because of their immunosuppressive properties and multipotency. In the present study, we examined the strategies for effective cell transplantation to develop a novel approach for functional recovery of the skeletal as well as cardiac muscles using a dog model of Duchenne muscular dystrophy. **Methods:** DPCs were intravenously injected into two littermates of canine X-linked muscular dystrophy in Japan ($CXMD_J$) at 2-week intervals for 10 times without immunosuppression. Clinical phenotypes in the transplanted dogs were analyzed by using blood exams, physical capacity, magnetic resonance imaging (MRI) analysis compared with non-injected littermates as controls. **Results:** There were no serious clinical events or adverse effects on DPCs-treated $CXMD_J$. The downregulation of inflammation in the lower legs of DPCs-treated $CXMD_J$ was confirmed by MRI analysis. Impaired tetanic force of gastrocnemius of $CXMD_J$ recovered in DPCs-treated $CXMD_J$, from 30% to 55% of wild type. Although $CXMD_J$ showed progressive muscle atrophy in the all four limbs, exercise intolerance and abnormal locomotion, we observed improved phenotypes in the DPCs-treated $CXMD_J$ along with the improved pace of flip-flop and running. **Conclusion:** We suggested that the systemic injection of DPCs ameliorated the progressive phenotype in $CXMD_J$. The therapeutic effects might be associated with the production of paracrine or endocrine factors that regulate inflammation, and might also stimulate the proliferation of endogenous stem cells at the injured muscle tissue. This strategy of DPCs treatment would be promising for the future DMD cell therapy.

424. Genome Wide Analysis of PAX7-Induced Myogenesis Identifies CD54 as a Novel Marker for cGMP-Compatible Purification of Human PS Cell-Derived Myogenic Progenitors

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Therapeutic application of pluripotent stem (PS) cell-derived products represents the ultimate goal of stem cell research. In order to apply this technology to patients, it is fundamental to characterize in detail the cell population of interest and identify strategies for its purification from unwanted cells using clinically-compatible methods. In the case of skeletal muscle wasting disorders, we have shown that human PS cell-derived PAX7-induced myogenic progenitors may represent an excellent candidate for cell therapy. To successfully translate this approach toward the clinic, we took advantage of next-generation sequencing techniques to dissect PAX7 function during human myogenesis. Combination of PAX7 genomic target profiling using ChIP-seq and whole transcriptome analysis (RNA-seq), in which we systematically evaluated different time points of the PAX7-dependent myogenic commitment from human PS cells, revealed a subset of genes differentially expressed at various stages of this differentiation process, including a discrete number of surface markers. After Fluorescence Activated Cell Sorting (FACS)-mediated screening, we identified α9β1 integrin, CD54 and Syndecan2 (SDC2), as potential surface markers to be used for the prospective isolation of human PS cell-derived myogenic progenitors. We demonstrate that these surface molecules reproducibly allow for the isolation of myogenic progenitors

from multiple human ES/iPS cell lines, in both serum- and serum-free culture conditions, and that α9β1+CD54+SDC2+ (triple+) cells represent a homogenous population of PAX7+ cells endowed with *in vivo* muscle regeneration potential. Furthermore, we demonstrate that a single marker is sufficient for the magnetic-based isolation of myogenic progenitors, thus enabling adaptation of our differentiation protocol to cGMP standards. These novel findings provide a clinically relevant method for the purification of PS cell-derived muscle progenitors for clinical applications.

425. TIPE2 Gene Transfer Attenuates Muscle Histopathology in *mdx* Mice

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Duchenne muscular dystrophy (DMD) is a deadly genetic disease mainly characterized by progressive weakening of the skeletal, cardiac, and diaphragmatic muscles. During healthy muscle repair, inflammatory responses are activated and are known to aid in the cleanup and restoration of damaged muscle. In DMD, however, these inflammatory responses are chronically activated and therefore become detrimental to the repair process. It is critical to find a successful therapy that will improve the histopathology of the muscles of DMD patients and restore their normal function. Many proinflammatory genes in DMD patients are upregulated within muscles beginning shortly after birth (Chen *et al.*, 2005). This early inflammatory response is followed in later stages by a failure of muscle regeneration with associated muscle fibrosis. In *mdx* mice, blockade of inflammatory mediators such as TNF-α and inducible nitric oxide synthase (iNOS) (Radley et al, 2008; Villalta et al, 2009) reduces muscle necrosis, suggesting that the host inflammatory response plays an important role in promoting muscle injury and subsequent fibrosis.

We posit that reducing muscle inflammation by regulating the expression of inflammatory mediators, would alleviate the progression of the disease in DMD; for example, preventing the excessive skewing of macrophages toward a proinflammatory phenotype can help restore muscle regeneration despite the absence of dystrophin (Mojumdar *et al.*, 2014). TNF-α-induced protein 8-like 2 (TIPE2 or TNFAIP8L2) is a negative regulator of innate and adaptive immunity and functions by maintaining immune homeostasis (Sun et al, 2008). To explore whether TIPE2 expression is diminished [L1] in the skeletal muscle of *mdx* mice (a mouse model of DMD), we performed immunohistochemical staining for TIPE2 expression on *mdx* muscle and found that TIPE2 expression is significantly lower in young *mdx* skeletal muscle when compared to wild type mice. To further investigate whether increasing expression of TIPE2 can improve muscle histopathology, we constructed an adeno-associated viral (AAV) vector encoding for TIPE2 gene under the CMV promoter and injected into 8-week-old *mdx* gastrocnemius muscle (GA); AAV9-CMV-GFP was also injected as control. Two weeks after injection, the mice were sacrificed and the muscles were sectioned. H&E staining revealed improved histopathology of the *mdx* muscle; there were more centrally nucleated muscle fibers (newly regenerated myofibers) in the AAV9-CMV-TIPE2-treated group than in the control group. Mouse IgG staining showed that there were fewer necrotic muscle fibers in the muscle injected with AAV9-CMV-TIPE2 than in the AAV9-CMV-GFP group. F4/80 (a macrophage marker) and trichrome demonstrated lower inflammation and fibrosis in skeletal muscles injected with AAV9-CMV-TIPE2 when compared to the AAV9-CMV-GFP control group. Our findings indicated that treatment with AAV9-CMV-TIPE2 resulted in decreased macrophage infiltration, fibrosis, and necrosis, while improving muscle regeneration in *mdx* mice. These results suggest that TIPE2 gene therapy could be a potential therapeutic approach for the treatment of DMD, and may help in the regulation of immune homeostasis in DMD patients.

Neurologic Diseases (including Ophthalmic and Auditory Diseases) II

426. Improvements in Aberrant Lipids and Mechanical Sensitivity After Intrathecal rAAV9-ABCD1 in a Mouse Model of AMN

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Adrenomyeloneuropathy (AMN) is the most common phenotype of X-linked adrenoleukodystrophy, a debilitating neurological disorder caused by mutations in the ABCD1 gene that encodes a peroxisomal ATP binding cassette transporter (ABCD1) responsible for transport of CoA-activated very long-chain fatty acids into the peroxisome for degradation. Close examination of the known Abcd1-/- mouse model of AMN reveals a 4-fold increase of C26:0 levels in spinal cord. Further we discovered that mechanical hypersensitivity of Abcd1-/- mice begins around 9 month of age, half a year prior to motor symptoms.

In previous work we demonstrated that rAAV9-mediated ABCD1 gene transfer via intrathecal osmotic pump (IT pump) led to more uniform and widespread gene delivery to CNS with reduced leakage into the systemic circulation compared to either intravenous injection or intrathecal bolus (IT bolus) injection. Here we report the biochemical and behavioral impact of intrathecal rAAV9-ABCD1 delivery.

rAAV9 encoding ABCD1 (rAAV9-ABCD1) were delivered to Abcd1-/- mice intrathecally (IT) at spine region L4-L5 using either a gas-tight Hamilton syringe attached to a 33-gauge steel needle over 2mins or an osmotic pump over 24h, with PBS injections serving as sham control. Two weeks after injection, mice were sacrificed and tissue was collected for lipid analysis (C26:0 measurements). For behavioral testing another cohort of mice was similarly injected with rAAV9-ABCD1 at 5 months of age.

Lipid analysis showed a 27% and 32% reduction in C26:0 of the spinal cord after rAAV9-ABCD1 IT pump and IT bolus injection ($1X10^{11}$gc/mouse) respectively. Importantly, after AAV9-ABCD1 delivery via IT pump we found a 2-fold improvement in the nominal force threshold compared to the PBS injected group. Behavioral testing after AAV9-ABCD1 IT bolus delivery is still pending. Meanwhile, no significant weight loss was detected after $1X10^{11}$gc rAAV9-ABCD1 IT pump delivery compared to the PBS group. Even a 3-fold higher dose ($3X10^{11}$gc/mouse) did not impact bodyweight, suggesting that intrathecal rAAV9-ABCD1 delivery is well tolerated.

We conclude that rAAV9-mediated ABCD1 gene transfer via intrathecal osmotic pump leads to biochemical correction and sensory improvements without associated toxicity. These findings may encourage further dose escalation and bode well for future clinical translation.

427. Safety of RGX-314 AAV8-anti-VEGF Fab Gene Therapy in NHP Following Subretinal Delivery

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Age-related macular degeneration (AMD) is the leading cause of blindness worldwide. 'Wet' AMD describes the abnormal growth of blood vessels that cause hemorrhaging and scarring of the central retina leading to blindness. The purpose of this study was to evaluate the safety and define an upper dose limit of subretinally delivered RGX-314 AAV8-anti-VEGF Fab as a treatment for wet AMD in non-human primates (NHP). *Cynomolgus* monkeys, ages 3-5 years, received a subretinal injection of 1e10 ('low', n=6), 1e11 ('middle', n=4) and 1e12 ('high', n=6) AAV8 GC/eye, or a control (n=4). Anti-VEGF Fab was assessed by VEGF ELISA of anterior chamber (AC) fluid. Retinal structure was evaluated with spectral domain optical coherence tomography (SD-OCT) at 3 (low and high doses) or 7 (middle) months post injection; function was evaluated with full-field electroretinography (ERG) (high and low-dose). Retinal distributions of anti-VEGF Fab mRNA and protein levels were also evaluated. At 30 days post injection there was a dose-dependent increase in anti-VEGF Fab expression: mean low dose =889 ng/ml (range 679-2116 ng/ml); middle=1505 ng/ml (431-2346 ng/ml); high =2452 ng/ml (966 - 3500 ng/ml); expression was not detectable in injected-controls. Anti-VEGF Fab mRNA and protein were distributed widely throughout the retina beyond the injected region. Retinal demelanization was observed in all injected regions, with no signs of intraocular inflammation. Low dose and control-injected eyes showed no significant difference in total retinal thickness (TRT) relative to vehicle-injected or uninjected controls, but there was significant TRT thinning within the injected regions in high (by 9%) and middle (24%) doses. There was outer nuclear layer (ONL) thinning only in the high (33%) and middle (18%) doses, but ellipsoid band (EZ)-to-Bruch's membrane (BM) shortening within the injected regions in all dose groups (high dose=51%, middle=20%, low=17%); no significant changes were observed in vehicle-injected compared to uninjected (99 percentile limits for change: TRT=14%, ONL=18%, EZ-BM=10%) eyes. Histopathology at 3 months showed minimal inflammation in the choroid in the low dose and inflammation of the retina, choroid, and sclera in the high-dose; at 10 months, 1 out of 4 eyes (2 NHPs) in the middle dose showed inflammation by histology. Vehicle-injected and low-dose groups did not show measurable ERG changes post injection, but there was a significant ($p<0.05$) decline in ERG amplitudes in the high dose compared to baseline, control-injected, and low dose. This study demonstrated expression of anti-VEGF Fab in AC at 30 days in all RGX-314 doses, as well as the feasibility of using OCT and ERG as outcome tools to define a safe dose in NHP dose escalation studies.

428. Comparison of Routes of Administration to the CNS for AAVrh.10-Mediated APOE2 Treatment of Alzheimer's Disease

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Alzheimer's disease (AD), an untreatable progressive degenerative central nervous system (CNS) disorder, affects 5.5 million Americans and is increasing in prevalence and economic impact. The major genetic risk factor for late onset AD are inherited variants of apolipoprotein E (APOE). Of the 3 common 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. Based on this data, we hypothesize that genetic modification of the CNS of E4 homozygotes to express the protective E2 allele would prevent progressive neurologic damage. In this context, we have demonstrated APOE2 mediated efficacy in an AD E4 transgenic (APP.PS1.TRE4) mouse model with a AAVrh.10 serotype vector encoding the human APOE2 cDNA (AAVrh.10hAPOE2) [Zhao L, et al, *Neurobiology of Aging* (2016)]. In the next step in moving APOE2 gene therapy for AD to the clinic, we evaluated the most effective, safe route of the AAVrh.10 vector delivery to mediate widespread distribution of therapeutic levels of the APOE2 protein in the brain of nonhuman primates (NHP). AAVrh.10hAPOE2-HA, an AAVrh.10 serotype coding for an HA-tagged human APOE2 cDNA sequence, was administered by 3 different routes to the CNS of African Green NHP (n=15), including (1) direct intraparenchymal to 3 sites in the hippocampal region ($5x10^{12}$ genome copies (gc) total); (2) intracisternal (IC, cisterna magna; $5x10^{13}$ gc); or (3) intraventricular (ICV, frontal horn of the 3rd ventricle; $5x10^{13}$ gc). At 13 wk (n=3-4/group) post-vector administration, the CNS was subdivided into 1 cm^3 cubes and quantitatively evaluated for APOE2 DNA, mRNA, and protein. The direct hippocampal route with $5x10^{12}$ gc provided high local concentration of APOE2 protein, as shown by immunohistochemical detection in the hippocampal/entorhinal region. At $5x10^{13}$ gc, the IC and ICV routes showed significant levels of vector copy number, APOE2 mRNA, and protein expression throughout the CNS including the hippocampal regions. General

safety, hematologic, serum chemistry, and blinded videotape analysis of behavior parameters were assessed over the course of the study with all routes; the vector-administered groups did not differ from the sham control in any parameter. While good hippocampal/entorhinal expression can be achieved with direct administration, there is a theoretical higher risk to safety of direct administration to this region. IC or ICV administration of AAVrh.10hAPOE2-HA at $5x10^{13}$ gc leads to broad, widespread distribution of vector, transgene, and APOE2 protein throughout the CNS, suggesting either the less invasive IC or ICV routes would be suitable for human administration.

429. Gene Therapeutic Correction of Sandhoff Disease in Neonatal and Adult Mice Using a Hexosaminidase Hybrid Neonatal and Adult Mice Using a Hexosaminidase Hybrid

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G_{M2} Gangliosidosis are neurodegenerative disorders caused by Hexosaminidase A enzyme (HexA) deficiency. HexA is the only enzyme in humans capable of catabolising G_{M2} gangliosides (GM2), where a HexA deficiency can lead to GM2 accumulation and widespread neurodegeneration within the central nervous system. HexA consists of α- and β- subunits, which when mutated cause Tay-Sachs disease (TSD) and Sandhoff disease (SD), respectively. In previously published work, a hybrid subunit was constructed which integrates the catalytic properties of the α-subunit and the stabilization and GM2AP binding sites of the β-subunit. This hybrid subunit, μ, coded by *HEXM*, homodimerizes to form the HexM enzyme, which can interact with GM2AP and catabolize GM2, effectively replacing HexA. In the current study, which is an extension to the limited data presented at last year's meeting, a self-complemetary adeno-associated virus 9 construct incorporating the *HEXM* transgene, scAAV9/*HEXM,* was intravenously injected into adult and neonatal SD mice to assess the effectiveness at different ages of administration. We injected 2.5E14vg/kg dose in neonatal mice. In the adult treated group, we investigated the use of two separate doses of treatment, 5E14vg/kg (high dose, HD) and 1.25E14vg/kg (medium dose, MD). We hypothesized that the HEXM treatment would significantly improve survival, locomotion, and biochemical outcomes in the SD mice. Although untreated SD mice survive only until 16 weeks of age, the neonatal, adult HD, and adult MD treated SD mice survived significantly longer, an average of 44.7, 58.8, and 40.6 weeks, respectively, with the oldest mouse surviving to 56.3, 89, and 62 weeks of age, respectively. Biochemical data shows that there is an increase in enzyme activity and a decrease in GM2 storage in the scAAV9/*HEXM* treated mice as compared to untreated SD mice. In addition, the behavioural data showed that HEXM treated mice perform better than untreated controls. Results from this study demonstrate a solid proof of corrective abilities of scAAV9/*HEXM* vector when administered in neonatal and adult SD mice. Histological and molecular analyses are ongoing. These results provide a step forward for human translation and a justification for consideration of a future gene therapy clinical trial using this vector for TSD and SD.

430. Systemically Delivered CNS-Directed Canavan Disease Gene Therapy at Different Ages Targets Brain Regions and Corrects NAA Metabolism at Similar Efficiencies but Restores Motor Function in an Age-Dependent Manner

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Canavan disease (CD) is a severe inherited leukodystrophy caused by autosomal recessive mutations in the aspartoacylase (ASPA) gene leading to accumulation of N-acetylaspartate (NAA) and spongy degeneration of the central nervous system (CNS). The majority of patients used to succumb to the disease in early childhood. However, with improving palliative care, a growing group of CD patients reaching adolescence and adulthood, leaving them behind of every effort to treatment development. We selected the Nur7 mouse model with a single point mutation in the *ASPA* gene and near normal life expectancy but frank neurologic phenotype and hypothesized that rAAV delivery in adult mice limits pre-clinical gene therapy in CD. We treated Nur7 mice intravenously as neonates (Neo), juveniles (Juv), adult (Adt) and mature adults (mAdt) and observed that their growth and cognitive function were all normalized or closer to wild-type (WT) mice. We assessed the animals for motor function by rotarod testing, starting 4 weeks post-treatment until the age of 1 year. The Neo and Juv groups performed as well as WT mice. While rotarod scores of the Adt group were significantly better than Nur7, mAdt mice did not show immediate response. A more sensitive CatWalk gait analysis, however, showed that even mAdts performed significantly better than Nur7 mice. Interestingly, all treatment age groups showed normalized signal intensities and NAA levels on T2 magnetic resonance imaging (MRI) and spectroscopy (MRS), respectively. In a similar trend, all the treatment age groups showed normalized neuropathology or remarkable mitigation of spongy degeneration. Moreover, analysis of myelination in the Juv group by electron microscopy (EM) revealed complete remyelination at 4 weeks post-treatment. We speculated that reduced rAAVhASPA CNS transduction might explain the limited response on motor function in mAdt mice. To address this concern, we analyzed 11 CNS regions by digital PCR but found no difference in rAAV genome abundance among all treatment age groups, suggesting that rAAV transduction is not the limiting factor and a window for effective treatment determines the therapeutic outcomes. In summary, regardless of treatment ages, rAAV-mediated systemic gene delivery to the CNS of Nur7 mice can target different regions of the CNS, correct NAA metabolic error, normalize the growth and restore cognitive function at similar efficiencies. However, extent of neuropathology mitigation, remeylination and motor function recovery varies significantly, ranging from complete resolution of neuropathology, full remyelination and restoration of motor functions in Neo and Juv groups to moderate improvement of neuropathology and motor

functions in Adt and mAdt groups. In order to achieve the maximum benefit of gene therapy in older Canavan disease patients, we are currently attempting to further define and extend the therapeutic window by exploring pathomechanism, additional disease modifiers and alternative biomarkers of CD.

431. SIRT1 Gene Transfer Promotes Retinal Ganglion Cell Neuroprotection in Experimental Optic Neuritis

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Multiple sclerosis (MS) is a chronic, inflammatory disease characterized by activation of $CD4^+$ T cells that infiltrate the central nervous system (CNS) and mount an autoimmune response against myelin. Optic neuritis (ON) is a condition commonly observed in MS patients that leads to temporary or permanent visual decline following demyelination of the optic nerve and loss of retinal ganglion cells. Current therapies for MS and ON include immunosuppressive agents that mitigate the inflammatory component of disease. Unfortunately, these treatments provide temporary symptomatic relief and, moreover, do not attenuate further neuronal loss. Therefore, it is critical to identify alternative treatment strategies that address underlying mechanisms of neuropathology. The conserved role of oxidative injury in MS and other forms of neurodegenerative disease is an attractive therapeutic target to delay or halt disease progression. Here we describe an approach that preserves retinal ganglion cell numbers and function during experimental optic neuritis following SIRT1 gene augmentation. SIRT1 is an NAD-dependent deacetylase that activates numerous cytoprotective mechanisms that suppress ROS activity, improve mitochondrial function, and inhibit apoptosis. We generated and characterized adeno-associated virus (AAV) vectors that drive constitutive expression of human SIRT1 using retinal-derived cell lines and *in vivo* models. Wild-type mice received intravitreal injections of AAV-SIRT1, AAV-eGFP, or vehicle. Afterwards, mice were vaccinated with myelin antigen to induce experimental autoimmune encephalomyelitis (EAE), an established model of MS that recapitulates the clinical features of optic neuritis including reduced visual acuity, optic nerve atrophy, and death of retinal ganglion cells (RGCs). Ganglion cell function was evaluated following EAE induction by measuring the optokinetic response (OKR). All EAE induced animals exhibited severely reduced OKR scores compared to sham immunized controls. However, treatment with AAV-SIRT1 improved visual acuity compared to vehicle and reporter injected animals also subjected to EAE. To examine the effect of SIRT1 gene augmentation on neuronal survival, retinae were harvested and stained with antibodies to label and quantify ganglion cells. Treatment with AAV-SIRT1 increased RGC survival compared to vehicle and reporter treated controls. Ongoing experiments will examine the contribution of SIRT1 gene augmentation in mitigating optic nerve atrophy as well as restricting oxidative damage in affected cell types. Collectively, this investigation suggests SIRT1 gene transfer can mediate neuroprotective effects in ON and MS pathogenesis.

432. Improved Targeting and Transduction Efficiency in the Central Nervous System After Systemic Delivery of an AAV-Derived Vector

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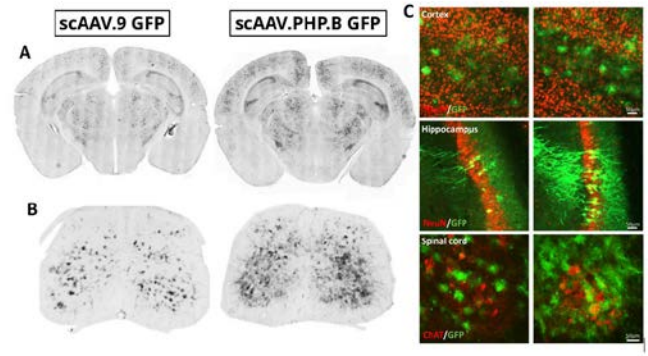
Introduction The success of any systemic viral vector delivery strategy targeting the central nervous system (CNS) is limited by the blood-brain-barrier (BBB), which prevents vector entry into the brain from the systemic circulation. Adeno-associated virus serotype 9 (AAV9) is considered the serotype with the highest tropism for CNS cells after systemic delivery. However, the efficiency of transduction and gene expression remains low. In this study, we attempted to improved CNS transduction using the recently described PHP.B capsid, in combination with a self-complementary (sc) genome enabling GFP expression under control of the widely used CBA promoter after systemic delivery.

Methods This approach employed a hybrid trans-complementing construct, encoding the Rep gene from AAV2 and the Cap gene from AAV9, or the recently reported PHP.B AAV variant, to produce a scAAV-based vector. Adult female C57/Bl6 mice (approx. 6 weeks of age weighing 20g) were administered a single intravenous dose of 1 X 10^{12} viral genomes of scAAV.9-CBA-GFP or scAAV.PHP.B-CBA-GFP. Additionally, two other groups of mice were stereotaxically administered scAAV.9 or scAAV.PHP.B into the lateral ventricle. Mice were euthanized 3 weeks post-injection and tissues collected for histological analyses.

Results The scAAV.PHP.B-based vector achieved widespread CNS transduction and higher levels of transgene expression following intravenous delivery when compared to the scAAV9 vector **(Figure 1)**.

Figure 1



Brain **(A)** and spinal cord **(B)** sections were immunostained for GFP and show widespread distribution and high expression of the transgene in the CNS. **(C)** Double immunostaining with NeuN or ChAT (red) confirmed that both neuronal and non-neuronal cells were transduced with scAAV9 (left panels) and scAAV.PHP.B (right panels).

Increased levels of expression, measured by fluorescence intensity, were significantly observed in all CNS regions in the scAAV.PHP.B group (*Brain:* 2105 ± 161 vs 1441 ± 99 au., *Spinal cord*: 2359 ± 267 vs 1639 ± 113 au. $p \leq 0.05$). In addition, cell-type tropism was observed. In cerebral cortex, more efficient astrocyte transduction was achieved with scAAV.

PHP.B vector delivery (37.9 ± 7.5 vs 12.8 ± 3.1 % of S100B/GFP+ cells; p≤0.001). No difference in transduction or transgene expression was observed between the 2 tested serotypes after intracerebroventricular delivery, suggesting increased BBB crossing efficiency of the AAV. PHP.B capsid as the reason for our findings.

Conclusions This result is the first report of the new PHP.B capsid used in the context of a scAAV vector. It underscores the potential of this AAV construct as a basis for more efficacious and safer viral gene therapy vectors in CNS, with important implications for gene therapy of neurodegenerative diseases. Additional transcriptional modifications, such as specific CNS promoters, or the use of enhanced regulatory modules, are necessary for further improvements of vector specificity and strength.

433. IND-Enabling Studies En Route to a Gene Therapy for *GUCY2D* Leber Congenital Amaurosis (LCA1)

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Mutations in *GUCY2D* are associated with severe early-onset retinal dystrophy, Leber congenital amaurosis type 1 (LCA1), a leading cause of blindness in children. *GUCY2D* encodes retinal guanylate cyclase-1 (retGC1), a protein expressed in photoreceptor outer segments that plays a role in the recovery phase of phototransduction. Despite a high degree of visual disturbance, LCA1 patients retain substantial photoreceptor laminar architecture suggesting that they are good candidates for gene replacement therapy. We have established proof of concept for AAV5-mediated *GUCY2D* replacement in mouse models of LCA1 and performed a preliminary dose response of AAV5-hGRK1-eGFP in non-human primate (NHP) to establish the dose required for efficient photoreceptor transduction. Here, we conduct additional preclinical studies to determine the minimum dose required in NHPs, evaluate systemic biodistribution of AAV5 following subretinal injection in a GLP study performed in rats, evaluate safety in a GLP toxicology study performed in NHPs, and confirm potency of the GLP material in retinal guanylate cyclase deficient mice. Cynomolgous monkeys were subretinally injected with a range of doses of AAV5-hGRK1-eGFP with contralateral eyes serving as uninjected controls. Spectralis imaging and immunohistochemistry of retinal sections were used to document GFP expression in life and to assess the percentage of photoreceptors transduced in the bleb area, respectively. Long Evans rats were subretinally injected with AAV5-hGRK1-GUCY2D at a high and low dose. Uninjected rats served as controls. Quantification of vector genomes in recovered tissue was assessed by qPCR. A formal GLP safety study is underway in Cynomolgous monkeys wherein a range of doses of AAV5-hGRK1-GUCY2D was delivered by subretinal injection. In-life ophthalmologic exams, OCT, ERG and histopathology will be used to assess safety. GC1/GC2 double knockout (GCDKO) mice were subretinally injected with either the same GLP test article used in biodistribution and safety studies or our prior research grade material at various doses. Four weeks post-injection, retinal function and structure were analyzed by ERG and OCT, respectively. The results of these studies expand on our knowledge of the dose response for an AAV5-hGRK1 delivered transgene in NHP with respect to both photoreceptor transduction and safety and, in combination with the rat biodistribution and mouse efficacy studies, will inform the design of a first-in-human clinical study in LCA1 patients.

434. Safety of XT-150 Human IL-10v Plasmid Gene Therapy Intrathecal in a Mouse Neuropathic Pain Model

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Chronic neuropathic pain (NP) is a debilitating condition that affects millions of patients world-wide. It results from multiple etiologies that lead to the impairment or damage of sensory neurons along the pain pathway: chemotherapy, neurotropic viruses, tumor growth, trauma, etc. Current therapies for the treatment of NP, directed at the neurons in the pain pathway, fail to treat the most serious conditions and are fraught with side effects that limit their use. Emergent evidence from multiple laboratories has established that a significant component in the establishment and prolongation of NP are activated glial cells that play a critical role in pain neuron function. We are developing glial-targeted pain therapies to treat NP, in particular human IL-10-variant-encoding plasmid-based gene therapies for the treatment of NP and other conditions. Here we describe the results of a GLP toxicology study examining two different formulations of this plasmid-based therapy in a mouse NP model. We demonstrate that these formulations drive the reversal of NP in rodent models for up to three months following a single intrathecal injection. We demonstrate that these therapies are well-tolerated with no toxicological findings, and that the aqueous XT-150 formulation has clear advantages over the encapsulated XT-101 formulation.

435. Extensive Dose Ranging Study of AVXS-101 in the Severe D7 SMA Mouse Model

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Spinal muscular atrophy (SMA) is a devastating, monogenic neurodegenerative disease, and the number one genetic cause of infant mortality. Gene therapy shows great promise for clinical use in

monogenic diseases, such as SMA type 1, as this approach addresses the root cause by providing a functional copy of the defective gene. Preclinical and clinical studies thus far have shown that adeno-associated viral vectors (AAV) specifically demonstrate positive safety profiles and efficacy. Here we report on an extensive dose ranging study of an AAV9 mediated gene therapy for SMA (AVXS-101) in the severe Δ7 SMA mouse model.

AVXS-101 utilizes the AAV9 vector which can cross the blood-brain-barrier and reach the critically affected cells in the central nervous system (motor neurons) as well as reach peripheral tissues which may also be contributing to disease symptoms. The AAV9 vector delivers a healthy copy of the human Survival Motor Neuron transgene. In this study, an extensive dose ranging was performed including ineffective, minimally therapeutic, intermediate, proposed therapeutic, and maximal doses. AVXS-101 was delivered via a single vascular injection to postnatal day 1 mice. The mice were dosed at postnatal day 1, as multiple pre-clinical studies have shown that optimal phenotypic rescue in the severe SMA mouse model occurs with early dosing, before extensive disease damage has occurred. Survival, body weight and motor function were evaluated to determine phenotypic rescue.

All animals tolerated the injection well and no signs of toxicity were noted for any dose. All doses above the ineffective dose demonstrated some level of phenotypic rescue with comparable levels of phenotypic rescue observed for the proposed therapeutic and maximal doses. Motor neurons were efficiently transduced along all regions of the spinal cord and efficiency increased with higher doses.

In conclusion, these results further support the clinical development of a single intravascular dose of AVXS-101 with a potential to be transformative in the treatment and management of Spinal Muscular Atrophy Type 1.

436. Gene Delivery of Vasostatin via a Self-Complementary Adeno-Associated Virus 2 Inhibits Ocular Neovascularization in Rats

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Ocular neovascularization is a common pathological feature in diabetic retinopathy and neovascular age-related macular degeneration, leading to severe vision loss. We evaluated the efficacy of a novel endogenous inhibitor of angiogenesis, vasostatin (VS180) and its functional fragment of 112 residues, vasostatin-like peptide 112 (VS112) delivered using a self-complementary adeno-associated virus 2 (scAAV2) in a rat models of oxygen-induced retinopathy (OIR) and laser-induced choroidal neovascularization (CNV). Expression of VS112 or VS180 was elevated in cells infected with scAAV2-VS112 or scAAV2-VS180, respectively, and both inhibited angiogenic activity in *in vitro* human umbilical vein endothelial cells. Retinal neovascularization (RNV) was induced by exposing newborn rats to 80% oxygen for 21 hours followed by room air for 3 hours every day during the first 14 days of life. Animals were returned to room air for another 4 days. Viral vectors were intravitreally injected at postnatal day 7 (P7) and eyes were harvested on day 18 (P18) for quantification of retinal vaso-obliteration and neovascularization, respectively. CNV lesions were induced in rat eyes by laser photocoagulation, and viral vectors were intravitreally injected immediately after laser injury. ScAAV2-VS112 or scAAV2-VS180 gene delivery significantly inhibited OIR-induced RNV in rat eyes (VS112: 1.29 ± 0.08%, VS180: 1.38 ± 0.11%) compared to the scAAV2-mCherry vehicle (2.62 ± 0.18%, $P<0.001$). Unexpectedly, there was a significant increase in OIR-induced vaso-obliteration in eyes that had received scAAV2-VS112 (39.7 ± 4.3%) compared to those injected with scAAV2-mCherry (19.9 ± 4.3%) or scAAV2-VS180 (22.3 ± 4.3%). Moreover, intravitreal scAAV2-VS112 and scAAV2-VS180 also significantly inhibited laser-induced CNV lesions as measured via choroid flat-mounts (day 28) compared to scAAV2-mCherry (VS180: 27.5% reduction, $p<0.05$; VS112: 44.0% reduction, $p<0.001$). Our data show that a single intravitreal injection of scAAV2-VS112 and scAAV2-VS180 inhibits ischemia-induced RNV and laser-induced CNV in rats. Thus, gene therapy using scAAV2-VS180 or scAAV2-VS112 has significant potential as a therapy for ocular neovascularization.

437. Using Deep Brain Stimulation Surgery as a Platform for Cell Therapy Delivery in Patients with Parkinson's Disease

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Clinical trials with gene or cell therapy focusing on neurodegenerative diseases face many hurdles. Issues from regulatory approval to patient well-being may short-circuit the trial even before questions related to efficacy can be addressed. Over the last several years, we have developed a strategy to address many of these issues and are in the process of carrying out two Phase I studies examining the safety and feasibility of autologous peripheral nerve grafts delivered to target brain areas at the time of deep brain stimulation (DBS) surgery in patients with Parkinson's disease (PD). DBS therapy is FDA approved for the treatment of several conditions including PD. We chose peripheral nerve tissue as donor material, because Schwann cells, after injury, transdifferentiate to become "repair cells" and release a host of factors including GDNF, NGF, BDNF, and NT-3. Here we describe how our cell therapy delivery strategy has helped us clear many of the hurdles encountered in early-stage clinical trials. Three key advantages of our design are 1) because participants have their own sural nerve removed and transplanted at the time of DBS surgery, without any significant modifications, the delivery of the grafts does not require FDA oversight; 2) because DBS surgery is an insurance reimbursable procedure, trial costs are greatly reduced, and 3) because DBS is a standard of care for Parkinson's disease, patients do not have to forego the therapeutic benefits of DBS to participate in the trial. To date, we have transplanted grafts into the substantia nigra and/or nucleus basalis of Meynert in

over 30 participants without any severe adverse events related to the grafting procedure. While dedicated efficacy studies are needed to fully assess the potential of this therapy, we are finding that combining the delivery of cell therapy at the time of DBS surgery provides substantial advantages for designing clinical trials examining biological therapies requiring surgical delivery.

438. Evaluation of *In Silico* Reconstructed Ancestral Adeno-Associated Virus for Gene Augmentation Therapy in a Mouse Model of LCA

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The Retinitis Pigmentosa GTPase Regulator Interacting Protein 1 (RPGRIP1) is an essential protein for the maintenance of photoreceptor outer segments. *Rpgrip1* knock-out mice have outer nuclear layer (ONL) thinning at 3 months of age, and nearly complete loss after 5 months. In humans, *Rpgrip1* mutations manifests as severe retinal dystrophy, known as Leber Congenital Amaurosis (LCA). Recently, our laboratory has shown for the first time, predicted protein sequences used to derive functional synthetic adeno-associated viruses (AAVs). One of the AAVs of interest, termed Anc80, is a potential putative ancestor of AAV serotypes 1, 2, 8 and 9. Anc80 is a highly potent *in vivo* vector with the ability to transduce various cell types such as, but not limited to, the liver, retina and muscle. Transduction levels with Anc80 are significantly superior to AAV2 and AAV8; two of the most commonly used gene therapy vectors to date. This study evaluated the use of Anc80 and AAV8 for gene augmentation therapy in *Rpgrip1* knock-out mice.

Firstly, Anc80.CMV.EGFP and AAV8.CMV.EGFP were subretinally injected into wild-type C57BL/6J mice to assess cell transduction patterns by fundus imaging and immunofluorescence (IF) analysis. Subsequently, *Rpgrip1* knock-out mice were treated with sub-retinal injections of either Anc80.RK.hRPGRIP1 or AAV8.RK.hRPGRIP1 at two weeks of age. Injections were performed in one eye, as the contralateral was used as an internal control. Expression of the *hRPGRIP1* transgene was evaluated using western blot (WB), RT-qPCR and IF analyses, 3 weeks post injection.

After 3 days, fundus imaging showed GFP expression by subretinal injection of either Anc80.CMV.EGFP or AAV8.CMV.EGFP. The area of transduction continued to expand until 2 weeks, and remained consistent until 5 weeks. GFP expression intensified between 2 and 5 weeks. By IF, Anc80 sections showed transgene expression in the retinal pigmented epithelium (RPE), photoreceptor cells, Müller cells, inner nuclear layer (INL), and the retinal ganglion cell (RGC) layer. AAV8 injected eyes showed similar transduction, but only from the RPE through the ONL, with some Müller cells. In the gene therapy study, *hRPGRIP1* expression was evident in knock-out mice by use of either Anc80 or AAV8 by IF, WB and RT-qPCR.

By use of a broad CMV promoter, Anc80 showed robust expression within the outer retina, which extended into the inner retina. Anc80 also mediated *hRPGRIP1* expression within the connecting cilium by use of the photoreceptor specific promoter, Rhodopsin Kinase (RK). Efficacy of gene delivery will be determined at 20 weeks post-injection by OCT and ERG.

439. Comparative Phylogenomics of the Dystrophin/Utrophin Supergene Family Yields Improved Transgenes for Gene Therapy in DMD

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A major physiological function of dystrophin is the protection of muscle cell membranes from the mechanical stresses developed during forceful contraction. Although AAV vectors have emerged as promising vehicles for systemic gene delivery in Duchenne Muscular Dystrophy (DMD), their limited cloning capacity has focused attention on the design of miniaturized transgenes to encode low molecular weight substitutes for full length dystrophin. The majority of dystrophin's molecular weight is contributed by the protein's rod domain, composed of 24 tandem repeats of a triple helical domain. It has been widely assumed that all 24 repeats are necessary for full function of dystrophin, a concept embodied in the suggestion that the protein functions as a "shock-absorber" that needs to be a certain length to adequately dissipate mechanical forces. We have leveraged newly developed computational platforms for comparative phylogenomics and protein tertiary structural inference to revisit the issue of dystrophin and utrophin's conserved features. The resulting data support a novel hypothesis that unifies a set of clinical observations from patients with Becker Muscular Dystrophy. We propose a model in which dystrophin and utrophin transmit force longitudinally through amino acid side chains that strongly interact at sites where the loops from adjacent triple helices interdigitate. Genomic data support a reconstruction in which tandem duplications extended the rod domain of an ancestral dystrophin homolog, but subsequent sequence divergence has limited the interchangeability of adjacent triple helices. Our detailed analysis has identified a rare subset of opportunities for internal deletion that minimize the disruptive effect of juxtaposing divergent, incompatible amino acids. We present examples of recombinant proteins that have the potential for greatly improved functionality without increased immunogenicity, and provide striking results of detailed *in vivo* analysis in DMD models, data thus far entirely consistent with our working hypothesis. We also provide early data from a rigorous translational approach designed to expedite clinical application in DMD.

440. Beneficial Effects of Allogeneic Hematopoietic Stem Cell (HSC) Transplantation in a Mouse Model of Infantile Neuronal Ceroid Lipofuscinosis (CLN1); Rationale for Promising HSC Gene Therapy Approaches

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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. Although gene-therapy is a promising therapeutic option for these pathologies, insufficient delivery of PPT1 activity to the central nervous system (CNS) resulted to be an issue that hindered successful clinical application of the approaches tested by now. We previously demonstrated that reconstitution of brain-resident myeloid cell/microglia by the progeny of gene-corrected hematopoietic stem and progenitor cells (HSPCs) in myeloablated transplant recipients can generate a local source of the functional hydrolase in animal models and/or patients affected by other Lysosomal Storage Disorders. This same approach may represent a strategy to obtain widespread distribution of functional PPT1 in the INCL CNS. Here we exposed Ppt1 -/- mice (a mouse model of INCL) to an optimized conditioning regimen based on systemic administration of busulfan in order to foster efficient turnover of brain myeloid cells/microglia with the progeny of HSPCs isolated from Ppt1 wild type mice and injected systemically or intracerebroventricularly (ICV). The transplantation resulted in a sustained and long-lasting donor-cell chimerism in the CNS of Ppt1 -/- recipient mice. Importantly, transplanted INCL animals showed a milder disease progression with less severe motor impairment. Histological analyses revealed a widespread distribution of donor-derived microglia cells throughout the diseased brains, especially in the areas that are mainly targeted by the pathology (i.e. cortex, hippocampus, thalamus, cerebellum and spinal cord). In these CNS districts, the donor derived microglia cells displayed high positivity for autofluorescent material, although the overall level of CNS storage was significantly reduced in HSC transplanted as compared to untreated Ppt1 -/- mice. This may suggest an engagement of microglia cells carrying the functional Ppt1 in the detoxification of the storage and overall support to the neuronal microenvironment. Indeed, partial neuronal protection was documented at multiple sites. This constitute first formal demonstration of the potential for benefit of HSC-based approaches in INCL. Therefore, based on these promising results, we started a preclinical study aimed at testing the safety and efficacy of an HSPC gene therapy approach, based on transplantation of HSPCs from Ppt1-/- mice into busulfan-myeloablated Ppt1-/- recipients, upon cell transduction with a lentiviral vector expressing supraphysiological levels of the wild type human PPT1. Overall, through this strategy we expect that microglia cells derived from the progeny of transplanted cells will potentially ameliorate the disease phenotype, by releasing the functional enzyme, allowing prominent, sustained and widespread distribution in the CNS of INCL mice. The study is currently in progress.

441. Production of Neurotrophic Factors by Constitutively Active ras Homolog Enriched in Brain Transduction of Adult Neurons *In Vivo*: A Potential Therapeutic Strategy Against Neurodegenerative Diseases

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Although fully understanding of the etiology of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) is still insufficient and no therapy exists to block neurodegeneration in patients with AD and PD, one treatment area that has gained significant momentum is the use of various neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), even though there were some reports showing that neurotrophic factors such as GDNF and neurturin delivered in PD patients were ineffective for PD therapy. Our recent results showed that the activation of neuronal mammalian target of rapamycin complex 1 (mTORC1) by adeno-associated virus 1 (AAV1) transduction with a gene encoding the constitutively active form of ras homolog enriched in brain (Rheb) could induce neurotrophic effects *via* the production of neurotrophic factors such as GDNF and BDNF, resulting in the protection and restoration of adult neurons against neurotoxicity in animal models of neurodegenerative diseases. These observations suggest that the increase in neuronal mTORC1 activity by a specific gene delivery can give adult neurons the important ability to produce neurotrophic factors as therapeutic agents against neurodegenerative diseases, and that viral vector transduction with active Rheb may have a therapeutic value in the treatment of neurodegenerative diseases such as AD and PD. Correspondence: Sang Ryong Kim, Ph.D. School of Life Sciences, Kyungpook National University, Daegu, 41566, S. Korea. Phone: +82-53-950-7362. Fax: +82-53-943-2762. E-mail: srk75@knu.ac.kr Acknowledgements: This work was supported by grants from the Korea Healthcare Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI15C1928) and the National Research Foundation of Korea (NRF-2012R1A1A1039140).

442. Voluntary Running Triggers VGF-Mediated Oligodendrogenesis to Prolong the Lifespan of Snf2h-Null Ataxic Mice

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Exercise enhances cognitive function and slows progressive neurodegenerative disease. While exercise promotes neurogenesis, oligodendrogenesis and adaptive myelination are also significant contributors to brain repair and brain health. Nonetheless, the molecular details underlying these effects remain poorly understood. Conditional ablation of the *Snf2h* gene impairs cerebellar development producing mice with poor motor function, progressive ataxia and death between postnatal day 25 to 45. Here we show that voluntary running induced an endogenous brain repair mechanism that resulted in a striking increase in hindbrain myelination and the long-term survival of *Snf2h* cKO mice. Further experiments identified the VGF growth factor as a major driver underlying this effect. VGF neuropeptides promote oligodendrogenesis *in vitro*, while *Snf2h* cKO mice treated with full-length VGF-encoding adenoviruses obliterated the requirement of exercise for survival. Together, these results suggest that VGF delivery could represent a therapeutic strategy for cerebellar ataxia and other pathologies of the central nervous system.

Oligonucleotide Therapeutics II

443. Nuclear Non-Coding RNAs Regulate VEGF-A Expression and Are Secreted from Transduced Cells to Surrounding Tissue and Blood Circulation in Mouse Myocardial Infarction Model

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Our group has shown that vascular endothelial growth factor A (VEGF-A) can be up- or downregulated utilizing shRNAs directed at different targets in the murine VEGF-A promoter. The therapeutic potential of upregulating VEGF-A expression by shRNA has been shown *in vivo* in mouse hindlimb ischemia and myocardial infarction models. We believe, that surprisingly good therapeutic efficiency is explained by two factors: 1) These shRNAs mimic action of naturally occuring microRNAs regulating chromatin function, 2) small RNAs are secreted to neighboring cells by extracellular vesicles. For the hypothesis (1), we aimed to find microRNAs that target murine VEGF-A promoter. Using bioinformatic tools, we predicted two miRNAs, mmu-miR-466c and mmu-miR-669c, to have VEGF-A promoter binding sites. qRT-PCR analysis confirmed that these miRNAs are upregulated upon hypoxia, as well as the SFMBT2 gene from which these miRNAs are expressed (intron 10). Next generation sequencing of miRNAs in mouse endothelial cells, treated with hypoxia for 0, 2, and 24h and split into nuclear and cytoplasmic fractions, confirmed that miRNAs localize also to the cell nuclei. Further, the expression level changes of miRNAs in response to hypoxia were shown to occur only in the nucleus or cytoplasm for individual miRNAs. We also identified previously unknown miRNAs, which were typically enriched in the nucleus. Deletion of miR-466c from the cell genome using CRISPR abolished the upregulation of VEGF-A in response to hypoxia, but was restored by ectopic expression of miR-466c. We also performed targeted RNA sequencing of promoter-associated transcripts, SFMBT2, HIF1α, YY1 and VEGF-A in normoxic and hypoxic conditions. Based on these data we have built a model for the regulation of VEGF-A in hypoxia by ncRNAs. For the hypothesis (2), we used tranwell assay to analyse secretion of shRNA strands and found that mature shRNA-451 sense and antisense strands are efficiently secreted to cell culture medium. Still, neither C166 nor MS-1 cells were found to intake secreted shRNA-451 strands *in vitro*. However, when cells from transduced infarcted hearts were sorted for co-expressed GFP (positive and negative fractions), we detected mature strand of shRNA-451 also in GFP negative population by qRT-PCR analysis, suggesting that in infarcted mouse heart shRNA-451 transfer from cell to cell occurs, proposing that transfer may be cell type or tissue dependent. Mature form of shRNA-451 is also found in serum of MI mice that have received intra-myocardial injection of lentiviral vector expressing shRNA-451. Therefore, also tissue to tissue transfer of shRNAs is possible. Together, these results expand the roles of small RNAs in the regulation of gene transciption *in vivo*.

444. Therapeutic Delivery of microRNA Attenuates Ischemia-Induced Retinal Neovascularization via Regulation of the TGFβ and VEGF Pathways

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Dysregulation of microRNA (miRNA) expression has been implicated in the pathogenesis of retinal neovascularization, suggesting novel avenues for therapy. In this study, we sought to identify miRNAs that may serve as therapeutic targets of for retinal neovascularization using a rat model of oxygen-induced retinopathy (OIR). Rat pups were subjected to cyclic hyperoxia or normoxia (control) for 14 days and retinal RNA was isolated for miRNA profiling by next generation sequencing (miRNA-seq). Four miRNAs, miR-143-3p, miR-126-3p, miR-150-5p and miR-145-5p, were shown to be uniquely down-regulated in this rat model, suggesting that these miRNAs may play roles in the pathogenesis of ischemia-induced retinal neovascularization. To validate the therapeutic efficacy of the identified miRNAs, intravitreal injections of synthetic miRNA mimics (1μg) were administrated to rats with OIR at postnatal day (P) 14, followed by evaluation of the pathological retinal vascular remodelling at P18.

Intravitreal injection of synthetic miR-143-3p, miR-126-3p or miR-150-5p mimics significantly suppressed retinal neovascularization in OIR rats, compared with those receiving scrambled RNA. Bioinformatic analysis suggested that these dysregulated miRNAs and their angiogenesis-relevant target genes were collectively involved in retinal neovascularization via the TGFβ1 and VEGF pathways. These data suggest that therapeutic delivery of of miR-143-3p, miR-126-3p or miR-150-5p may represent a new approach for targeting retinal neovascularization in diseases such as proliferative diabetic retinopathy.

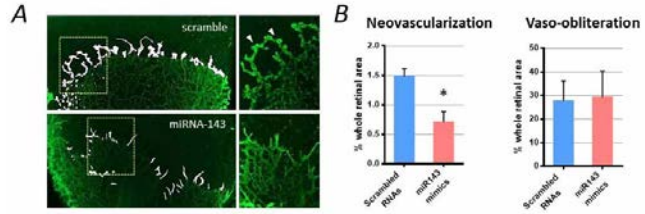

Figure. Intravitreal injection of miRNA mimics suppressed retinal neovascularization. (A) Representative images of OIR retinas following intravitreal injection of a miR-143 mimic or a scrambled RNA. (B) Quantification of pathologic neovascularization and vaso-obliteration in OIR rats treated with a miR-143 mimic in one eye and scrambled RNA in the fellow eye. MicroRNA-143 mimic treatment significantly supressed pathologic neovascularization with no significant difference in vaso-obliteration (N=3-5 per group). *p<0.05.

445. Overexpression of FKRP by Targeting with Oligonucleotide as Therapeutic for Dystroglycanopathy

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Dystroglycanopathies are characterized by the loss or reduction in functionally glycosylated a-DG (F-a-DG). Mutations in the gene for fukutin-related protein (FKRP) are the primary cause of limb-girdle muscular dystrophy 2I (LGMD2I) with muscle fibers lacking detectable F-a-DG. However, a small number of fibers expressing strong F-a-DG, referred to as revertant fibers, have been identified in muscle biopsies of patients with FKRP mutations as well as mutant mouse models. The presence of these fibers suggests, at minimum, a partial function of mutant *FKRP* and provides evidence for its use in restoring normal levels of F-a-DG. The overexpression of mutant *FKRP* may provide a viable mechanism for treating *FKRP* related dystroglycanopathies. Here we explore the use of antisense oligonucleotides (AON) for enhanced mRNA stability of *FKRP* as well as targeting of the promoter for RNA activation. We demonstrate increased expression levels of *FKRP* in the skeletal muscle following short-term and long-term treatments with vivo-PMOs targeting the 3' UTR of *FKRP* mRNA. Short term treatment targeting the promoter region of *FKRP* also results in increased expression levels as measured by quantitative-RT PCR. Our results indicate that upregulation of *FKRP* through AON targeting can be achieved *in vivo* and provides an alternative approach to gene overexpression in treating dystroglycanopathies.

446. Delivery of Chemically Stabilized MicroRNA for Treatment of Liver Fibrosis

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Extracellular matrix (ECM) proteins deposition is the characteristic feature of liver fibrosis. Hepatic stellate cells (HSCs) are considered to be the primary source of ECM. Excess production of ECM proteins in the liver serve as a barrier to efficient drug deliver to the liver. MicroRNA 29 (miR-29) family composed of miR-29a, b, and c is downregulated in fibrotic liver. Among them miR-29b plays an important role in liver fibrosis, because it targets several of ECM component proteins including collagens, elastin, and fibrillin, and several ECM promoter genes including Bromodomain-containing protein 4 (BRD4), tissue inhibitor of metalloprotease-1 (TIMP-1), heat shock protein 47 (HSP47) and lysyl oxidase (LOX). However, the use of miRNA in a clinically relevant scenario has not yet been demonstrated because of their inherent instability and rapid elimination from the plasma due to uptake by the reticulo-endothelial system and renal clearance. Formulation of miRNA into polymeric carriers can prolong its circulation time and prevent its degradation from RNases. However, currently available carriers fail to protect miRNAs from intracellular degradation. Backbone modification can protect miRNA from RNases without decreasing its efficacy. We have partially modified miR-29b1 at its antisense strand with phosphorothioate (miR-PS), 2'-O-methyl phosphorothioate (miR-OME), locked nucleic acid (miR-LNA), and N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine (miR-ZEN) at 3'-end and determined their stability as well efficacy. To determine the stability, these miRNAs were incubated (1μg) with 50% fetal bovine serum (FBS) for 0-24 h at 37°C, loaded onto 15% non-denaturing polyacrylamide gels containing ethidium bromide. The mean density of miRNA bands was quantified using ImageJ analysis software. The effect of chemical modifications of miR-29b1 on collagen, α-SMA, and FN-1 gene expression was also determined after incubating these miRNAs (33 nM) at 37°C with HSC-T6 for 48h in 6 wells plate. Further, to improve pharmacokinetic profile and cellular uptake, we loaded modified miRNA in our previously reported poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol-graft-tetraethylenepentamine (mPEG-b-PCC-g-DC-g-TEPA) cationic polymeric micelles. Micelles were characterized for particle size, kinetic stability, complex formation and miRNA release, and evaluated for cellular uptake and efficacy in-vitro. We demonstrated a significant improvement in miR-29b1 stability in 50% FBS by backbone modifications. miR-29b1 unmodified degraded within 30 min of incubation in FBS, while miR-PS and miR-OME miR-LNA, and miR-ZEN remained stable even after 24h. Among the modified miRNAs, dual modified (MiR-PS and miR-OME) miRNA showed the highest stability. We observed a significant decrease in collagen-1 and fibronectin-1 gene expression in HSC-T6 cells after transfection with all modified miRNAs, with no adverse effect of chemical modifications. Micelles carrying miR-29b1 were spherical in shape with the mean particle size of 60 ±10nm. Kinetic stability determined by FRET analysis suggested that micelles were stable up to 36h when incubated with serum. The miRNA containing micelles were taken up by nearly 90 % of the cells, as determined by flow cytometry. In addition, micelles were observed intracellularly, as shown by confocal microscopy. In conclusion, our studies hold clinical potential for enhancing the

efficacy of miRNA based therapeutics. Currently, we are testing these modifications in common bile duct ligated animal model of liver fibrosis, but our approach is equally applicable to other disease models.

447. miRNA in Cell Cycle Regulation, Nuclear and Mitochondrial Communication: A Possibility

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Objective: The advancement in omics technology has brought a paradigm shift in our view on genome and its regulation. MiRNAs which were otherwise known as post transcriptional gene silencer are now emerging with various non-canonical functions, including translation activation, new localization, gene regulation pre transcription by miRNA binding at promoter region, epigenetic regulation such as histone modification and methylation. The other possibility includes inter organelles communication and process regulators. In this study an attempt has been done to identify miRNA that could regulate and coordinate the complex process of cell cycle by interacting with gene promoter of Cyclins, CDKs and mitochondrial DNA (mtDNA) in parallel. **Method:** With the help of Perl programming, longest seed sequence matches between miRNA and promoter region of Cyclins, CDKs as well as mtDNA. The localisation of these were further analysed based on ASUS motif for nucleotides and previously known mitomiRs by deep sequencing, microarray and qPCR. By blocking the cell cycle in the different stages and introducing miRNA endogenously, mtDNA replication and communication between nuclear DNA and mtDNA will be investigated. **Results:** We obtained 5 miRNAs out of 2578 analysed miRNAs that have longest seed sequence matches with cell cycle regulator promoter region along with long seed sequence matches in mtDNA. These could be further analysed to identify the potential role they play in regulating inter cellular communication during the process of cell cycle. **Conclusion**: This study is the first of its kind directing the potential role of miRNA in organellar communication during cell cycle. These miRNAs can be potential candidate to control cell cycle aberration and reprogram intracellular communication.

448. RNAseH Mediated Allele-Specific Silencing of a Dominant Mutation in CollagenVI-Related Dystrophy

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Collagen VI-related dystrophies (COL6-RD) are a group of congenital neuromuscular disorders found worldwide, that encompass a wide phenotypic spectrum ranging from the severe Ulrich (UCMD) to the milder Bethlem myopathy and manifest as generalized muscle weakness, distal joint hypermobility, proximal joint contractures and respiratory failure. Collagen type VI is a multimeric protein comprised of three chains, α1, α2 and α3 collagen chains encoded by the *COL6A1*, *COL6A2* and *COL6A3* genes respectively. These chains participate in a hierarchical assembly process as intracellular monomers, dimers and subsequent tetramers that are secreted into the extracellular space to form the microfibrillar collagen matrix. UCMD is commonly caused by dominant-negatively acting mutations in any of the three *COL6* genes. Dominant-negative mutations are usually in-frame triple helical exon skips or missense mutations of the triple helical glycine, that allow the mutant chains to be carried forward in the assembly to the full tetramer, thereby amplifying their effect. Haploinsufficiency for a *COL6* gene on the other hand is not associated with a clinical phenotype. Achieving allele-specific silencing of the mutant collagen VI transcript as a therapeutic approach would convert this dominant-negative state into a clinically asymptomatic haploinsufficient state. We have previously provided proof of principle for this approach using siRNA oligonucleotides. Gapmer antisense oligonucleotides (ASO) are known to cause target-RNA knockdown utilizing the function of intracellular ribonuclease H1 (RNAseH1). This type of oligonucleotide is attractive as it is more advanced in clinical development for a variety of applications. In our lab, we have explored the possibility of utilizing RNAseH-competent ASO to cleave RNA in an allele specific manner. As an initial screen we designed phosphorothioate (PS) oligonucleotides and tested their ability to downregulate the expression of a mutant *COL6A1* transcript with a deletion in exon 14, in patient derived human dermal fibroblasts. We monitored knock down efficiency and allele specificity using semi-quantitative PCR, quantitative RT-PCR and confocal microscopy on treated fixed cells, and found that the PS ASOs caused a significant and specific knockdown of mutant transcripts in a dose dependent manner. Additionally, we observed a decrease in the dominant negative effect of the mutation as evidenced by the improved formation of collagen VI microfibrils in the matrix. This study provides further insight into the potential for allele-specificity of RNAseH to target dominant mutations at the transcript level, with the goal of developing optimal compounds for *in vivo* application.

449. Over Expression of miR-219

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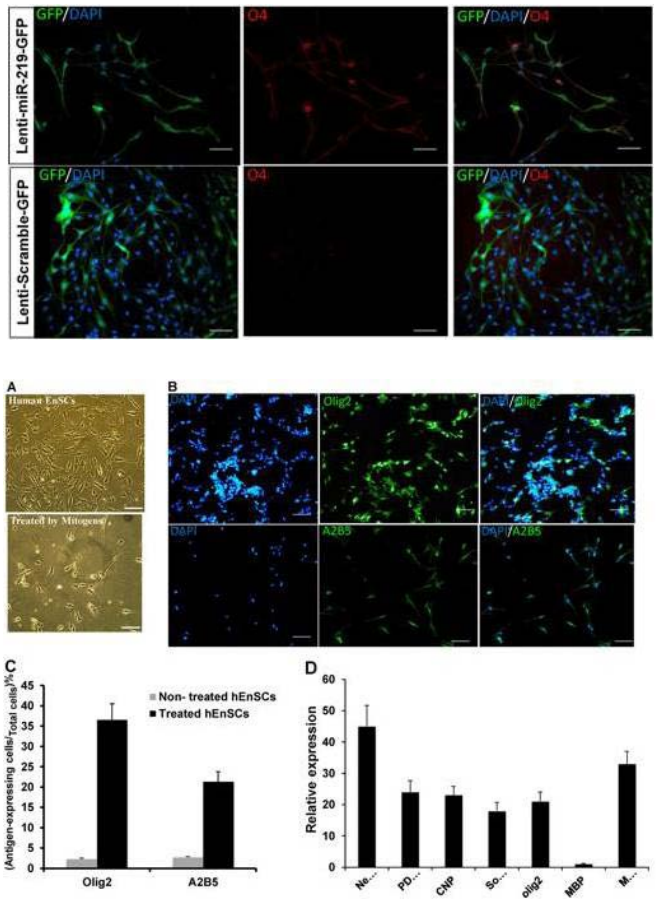
Abstract: Cell transplantation strategies are potential therapeutic approaches for treatment of neurodegenerative diseases by replacing of myelin-forming cells to induce re-myelination. Oligodendrocytes (OLs) are microglia cells that play a critical role in the central nervous system (CNS) by producing multilamellar myelin membranes for ensheating axons.MicroRNAs have critical roles in oligodendrocyte development.Several studies have shown that miR-219 is necessary to promote oligodendrocyte differentiation through repressing negative regulators of oligodendrocyte development. Human endometrial-derived stromal cells (EnSCs) are abundant and available adult stem cells with low immunological incompatibility, which could be considered for cell replacement therapy in future. After induction of EnSCs by FGF2,EGF and PDGF-AA, they were infected by miR-219-GFP-expressing lentiviruses. The cells were analyzed for expression of stage-specific oligodendrocyte cells markers. Quantitative RT-PCR and immunocytochemistry analyses showed that stage-specific markers Nestin, Olig2, Sox10, PDGFRa, CNP, A2B5, O4, and MBP

are expressed in their specific stages through differentiation protocol. Results showed that expression of pre-oligodendrocyte markers in miR-219-GFP-expressing cells were higher than triiodothyronine(T3) treated cells. In conclusion, the EnSCs could be programmed into pre-oligodendrocyte cells by overexpression of miR-219, and may convince to consider these cells as safe source for cell replacement therapy of neurodegenerative diseases.



450. Nuclease-Activated Oligonucleotide Probes for the Rapid and Robust Detection of Breast Cancer Circulating Tumor Cells (CTCs)

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Metastatic breast cancer is the second leading cause of female cancer deaths in the United States. Despite substantial progress in its treatment, metastatic breast cancer remains incurable. Early identification of breast cancer patients at greatest risk of developing metastatic disease is thus an important goal that would enable oncologists to aggressively treat these patients while the cancer is still vulnerable. In addition, this would spare patients who do not need or would not benefit from further treatments from having to endure the harmful side-effects of chemotherapeutic drug regimens. Circulating tumor cells (CTCs) are rare cancer cells found in the blood circulation of cancer patients that provide a non-invasively accessible cancer cell specimen (liquid biopsy) from patients. A challenge for CTC-based diagnostics is the development of simple and inexpensive methods that can detect these low levels of CTCs. The only FDA approved method for detection of CTCs is the CellSearch approach that captures CTCs by means of an antibody binding to epithelial cell adhesion molecule (EpCAM). However, this limits the detection to cancer cells of epithelial morphology, while those CTCs that in the metastatic course underwent epithelial mesenchymal transition (EMT) and express only little or no EpCAM will not be detectable. To overcome these limitations, we have developed a simple and inexpensive assay that detects CTCs with high sensitivity by measuring their nuclease activities. Advantages of nucleases as CTC biomarkers (*vs.* EpCAM) include their ubiquitous expression and their use to amplify detection signals. we have optimized the assay condition with respect to choice of probe, probe concentration, and buffer conditions (e.g. cations, pH). Our assay is based on quenched fluorescent nucleic acid probes that are selectively digested (activated) by nucleases expressed in breast cancer cells. The detection sensitivity was about 5 cancer cells with a 1-h assay. In addition to this, the best probe was found to give a linear correlation between signal and cell number down to one single cell. Furthermore, the assay shows robust performance in distinguishing blood samples of patients with breast cancer from those of healthy controls. Analysis of the data sets from Stage IV breast cancer patients and healthy donors yielded 61% sensitivity with 100% specificity for assay times as short as 20 minutes, exceeding the sensitivity of methods currently used in the clinic. This work may provide the foundation for robust point-of-care diagnostic tools for breast cancer and the approach may be useful in the diagnosis of other cancers. Future efforts will focus on evaluating the efficacy of the nuclease probe assay for early stage disease and as a point-of-care diagnostic.

451. Development of Polyglycan Specific Aptamers Against GP120 for Two-Stage Interference of HIV-1 Infection

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HIV *Env* protein gp120 non-covalently associates with gp41 forming heterodimeric complexes, which then further clustered into trimeric entry spikes and initiate HIV viral entry through contact with cell surface receptors. Besides, *Env* protein spikes are densely distributed on the surface of infected host cells during the final budding. Theoretically, agents targeting gp120 can be engineered to endow potencies against two stages within the viral infection cycle: entry and budding. Gp120 presents three distinct surface areas: **CD4BS** presents epitope directly against CD4 binding site thus blocking GP120-CD4 association; **CD4i** carries epitope induced or exposed only upon CD4 binding, blocking GP120-CD4 interaction to coreceptor; then the **silent face** that is highly glycosylated. Majority of the reported gp120 targeting antibodies and aptamers targets CD4BS and CD4i, hence the inhibitory efficacy against viral entry is largely limited by CD4 association; further due to the coplanar existence of gp120 and cell surface receptors, these agents generally fail to target the viral budding stage. Approximately half of the molecular mass of gp120 is contributed by N-linked polyglycans on the silent face, which shields gp120 from immune recognition, but also serves as potential epitopes

that are under-exploited. The unique configuration presented by the surface polyglycans renders rich hydrogen-binding patterns, thus suitable for aptamer selection. Aptamers developed to target the silent face is proposed to function allosterically and independent of CD4 or coreceptor binding. Unlike antibodies that present batch to batch difference and are lack of chemical modifications, aptamers carries definitive properties and are convenient to be chemically modified for increased stability and potency; further, aptamers can be couples with other chemical moieties for expanded function, such as drug/gene delivery and HIV diagnosis. To evolve aptamers that specifically target the surface polyglycans of gp120, gp120 protein is purified from human T-cells bearing close-to-native state glycosylation pattern. DNA library against gp120 polyglycan clusters is evolved for 10 cycles with increased selection stringency, and utilizing de-glycosylated gp120 as counter selection. The final selection pool is sequenced and analyzed. HIV challenge assays are performed to analyze the neutralizing effect presented by the selected aptamers. To follow up, lead aptamers will be extensively modified to bear artificial sugar backbone for prolonged physiological stability; photo-crosslinking functional groups can be displayed at aptamer contacting loops/bulges for activatable covalent association hence persistent inhibition; further aptamers can be conjugated onto branched polymers/dendrimers that can result in multivalent targeting. Further, selected aptamers can be incorporate into liposomes or lipidated micelles for site specific gene/drug delivery. Aptamers can be modified with fatty acids and embedded at the surface of lipidated delivery vehicles, enable site directed therapeutic delivery to infected host cells.

RNA Virus Vectors

452. Novel All-in-One LeGO-Tet Vectors: A Flexible Tool for the Analysis of Gene Functions by Conditional Expression

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Lentiviral vectors (LVV) have been widely used in basic research and gene therapy for the efficient delivery of genetic material and stable integration into the host genome. Whereas most LVV available confer steady transgene expression, a time- and dose-dependent regulation of expression has proven highly beneficial for detailed analysis of gene functions.

We have developed novel all-in-one LVV by combining our well-established platform of lentiviral gene ontology (LeGO) vectors with the Tet-inducible KRAB-domain containing tTR fusion repressor, which has shown superior Tet-responsiveness over the original tTA. The novel LeGO-Tet vectors were optimized for conditional expression by designing a variety of multi-cistronic expression cassettes and improving the position of the tetracycline response element. LeGO-Tet vectors represent versatile self-regulating LVV with the capacity to simultaneously transfer a gene of interest, a shRNA, a fluorescent reporter and a selectable marker in one single vector. Sensitivity of the fluorescent marker towards regulation was further enhanced by addition of a destabilization domain enabling combined control through Tet-dependent expression and protection from degradation. Unique cloning sites allow rapid exchange of vector modules and easy adaption to individual experimental conditions. Tet-controlled cDNA and shRNA expression with high-level induction and low background even in bulk cultures was demonstrated in various cell lines and primary cells, including mesenchymal stromal cells (MSCs) and keratinocytes. To further evaluate the functionality of the novel LeGO-Tet vectors, we analysed the conditional expression of a human oncogene (PIK3CA) that encodes the catalytic subunit of class IA PI3-kinase (PI3K). We demonstrated Tet-controlled expression of the wild-type PI3K and an activating mutant (H1047R) in IL-3-dependent haematopoietic cells (Ba/F3). Induced expression of mutant, but not wild-type PI3K resulted in a strictly Tet-dependent activation of the PI3K/AKT signalling axis. LeGO-Tet vectors enabled time- and dose-dependent oncogene expression and Tet-controlled regulation of cell proliferation, survival and IL-3-independent growth. To demonstrate efficient downregulation of gene expression in response to Tet withdrawal *in vivo*, we transplanted Tet-treated cells with confirmed high-level expression of the activated PI3K mutant into syngeneic mice (BALB/c) and monitored recipient mice for oncogene-induced leukaemic disease. Importantly, outgrowth of transformed cells was observed in all recipients under Tet treatment (10/10), but only in 1 out of 15 mice kept without Tet. The very low background activity of the LeGO-Tet vectors was confirmed in limiting dilution assays, where the frequency of IL-3-independent clonal growth was more than 10.000-fold lower in the non-induced compared to Tet-induced cells.

In conclusion, the novel LeGO-Tet LVV allow for controlled and titratable expression of a gene of interest in conjunction with fluorescent and selectable markers in a single vector and offer new possibilities to investigate gene functions by conditional expression both *in vitro* and *in vivo*.

453. DAPRins as Flexible Tool for Vector Retargeting

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From the portfolio of gene delivery vehicles, lentiviral vectors (LVs) and adeno-associated virus (AAV) vectors are currently most often applied in gene therapy. Delivering genes selectively to the therapeutically relevant cell type is among the prime goals of vector development. Vector tropism can be restricted at the level of cell entry through the display of targeting domains exhibiting high affinity for a cell surface protein characterizing the target cell type. The flexibility of this approach mainly depends on the availability of receptor specific targeting domains. In this context, we have described designed ankyrin repeat proteins (DARPins) which have been successfully displayed on the surface of LVs, AAVs and also oncolytic viruses. DARPins appear to be especially suited for this purpose since they have a compact highly stable α-helical fold. We generated a novel combinatorial DARPin library covering more than 10^{12} variants from which DARPins can be easily transferred to viral vectors. For selection, the extracellular

domains of target receptors are expressed in mammalian cells, purified and immobilized. Then, high affinity binders are identified from the DARPin library in several rounds of ribosomal display selection. This way, we have generated DARPins specific for the glutamate receptor subunit GluA4, the T cell marker CD8 and the NK cell marker NKp46. GluA4 is a marker for a subpopulation of interneurons which are strikingly diverse, constitute only a minority of all neurons in the brain and are therefore difficult to target. Selected GluA4-DARPins proofed to be specific for GluA4 and showed no cross-reaction to the closely related GluA2 receptor expressed on excitatory neurons. LV and AAV particles displaying GluA4-DARPins selectively transduced GluA4 expressing CHO cells. Taken together, the DARPin technology allows the generation of high affinity binders distinguishing closely related receptors as well as the corresponding receptor-targeted viral vectors exhibiting high selectivity for their target cells.

454. Baboon Envelope Pseudotyped Lentiviral Vectors: A Highly Efficient New Tool to Genetically Manipulate Healthy and Acute Lymphoblastic Leukemia T Cells

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Today, T cell receptor stimulation is still mostly used to allow efficient retroviral vector gene transfer, resulting in skewing of T cells towards a memory phenotype and compromising the longevity of these cells. However, T-cell based therapies would strongly benefit from gene transfer into live-long persisting T cells. In vivo persistence of gene modified T cells correlates with a less differentiated T cell phenotype maybe optimal in this setting. Therefore, we compared the gene transfer efficiency into more immature naive T cells with a new lentiviral vector (LV) pseudotyped with the baboon retrovirus envelope (BaEV) to other existing pseudotypes: VSV-G-LVs which is commonly used, RDTR-LVs, carrying the cat retroviral envelope and HF-LVs, displaying the measles virus envelopes. These BaEV-LVs outperformed the other LV pseudotypes by far for transduction of memory and naïve adult T cells upon IL-7 stimulation. Moreover, they out performed other LV pseudotypes by far for transduction (80-90%) of cord blood recent thymic emigrants upon IL-7 stimulation, allowing to conserve their naive phenotype, therefore they represent excellent tools for naive T cell transduction. The malignant transformation of normal T-cell progenitor cells into self-renewing leukemia-initiating cells causes the development of T-cell Acute Lymphoblastic Leukaemia (T-ALL), an aggressive hematologic cancer presenting unfavourable clinical features. Leukaemia-initiating cells are linked to chemotherapy resistance and relapse but the lack of tools to manipulate them prevents identification of their immunophenotypic and molecular features, thus precluding the development of novel targeted herapies. Our study reveals that BaEV-LVs are excellent tools to genetically manipulate T-ALL leukaemia initiating cells. These LVs enabled high-level transduction at low multiplicity of infection (MOI=10) in both healthy and malignant cells since they use ASCT1 and ASCT2, two amino acid transporters, which are highly expressed on the healthy T cells and T-ALL cells. The transduced blasts engrafted cohorts of primary and secondary NOD/SCID gamma mice, with typical T-ALL features, thus demonstrating that BaEV-LVs target leukaemia-initiating cells. Competitive xenograft models demonstrated that the BaEV-LV do not alter leukemic self-renewal, as the transduced blasts did not acquire a growth advantage, nor were they outcompeted by untransduced cells. Furthermore, our BaEV-LVs will facilitate chimeric antigen receptor expression and the development of genomic editing strategies for primary T-ALL cells. BaEV-LVs are thus excellent tools to genetically manipulate healthy T cells for therapy and malignant T cells to aid revealing novel therapeutic avenues.

455. Standardizing Clone Tracking Analysis and Characterization of Integration Profiles in Gene Modified Cell Populations

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Retroviral mediated gene therapy often targets hematopoietic stem cells (HSCs). The specific genomic location of retroviral integration sites (IS) can be used to identify and longitudinally track gene corrected clones in order to characterize the dynamics of reconstitution, the safety of the chosen vector, and the efficacy of treatments. Currently, the primary software packages used for IS analysis focus on extracting genomic locations from raw sequence data but lack the necessary tools to condense large tables into meaningful and interpretable graphical outputs. **Here we present a novel software package that not only extracts genomic locations of IS from raw sequence data but also provides the essential tools for annotating, condensing, and visualizing this data.**

We designed this software package to be highly customizable, compatible with a wide variety of sequencing methods, and interface seamlessly with existing software platforms. Multiple input formats are supported, and genome/vector sequences can be customized by the user in order to maintain relevance across models. Annotation and figure generation tools are compatible with outputs of existing IS analysis software. Annotation of IS informs the user on the insertion position relative to genomic features - which is crucial for understanding patterns of integration and allowing the user to assess the safety of a given treatment.

Figures corresponding to IS analysis generally cover four areas: 1) the diversity of IS, 2) clonal contributions over time or across cell/tissue types, 3) the distribution of integrations across the genome, and 4) IS position relative to genomic features. Here we propose standard representations of IS data and present tools for generating graphs corresponding to Shannon diversity indexes, bar charts depicting clonal frequency contributions, and probability density plots or frequency distributions of distances to various genomic features. We also present a tool that interfaces with Circos© to create various circular and 2D graphical genomic outputs such as line, scatter, heatmap, and histogram plots.

Using this software package, we characterized clonal repopulation dynamics after autologous bone marrow transplantation in 12 non-human primates with follow up periods between six months and seven years containing over >148,000 IS. Figure 1 depicts the overall lentiviral integration patterns of the pooled dataset as well as the diversity index and clonal contributions of one study animal.

Our software package provides a highly customizable pipeline for analysis of IS data, is compatible with various sequencing methods, supporting outputs obtained from existing analysis software, and provides tools for generating figures depicting IS diversity, clonal contributions, genomic integration patterns, and IS distances to genomic features which are essential to standardizing the evaluation of retrovirus integration in cellular DNA.

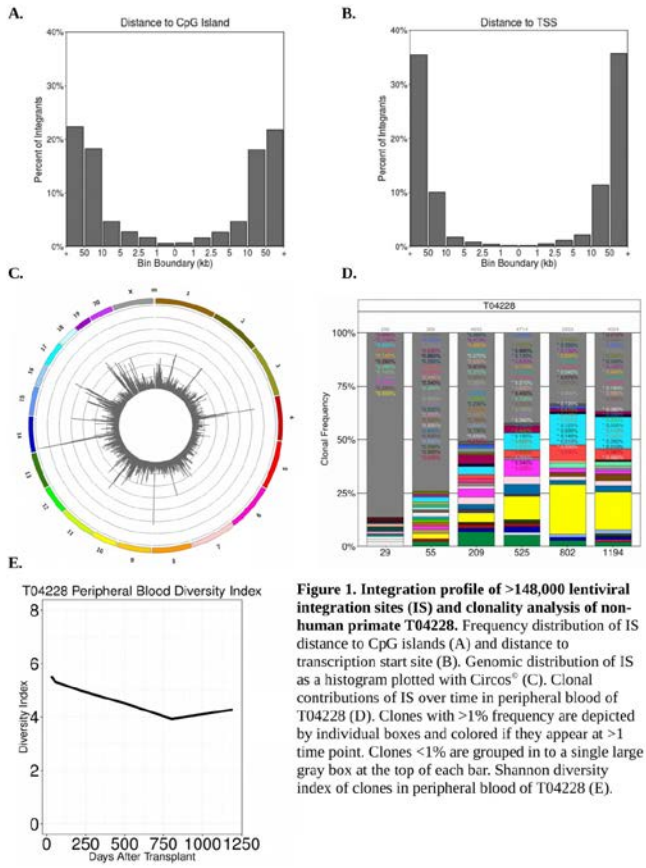

Figure 1. Integration profile of >148,000 lentiviral integration sites (IS) and clonality analysis of non-human primate T04228. Frequency distribution of IS distance to CpG islands (A) and distance to transcription start site (B). Genomic distribution of IS as a histogram plotted with Circos© (C). Clonal contributions of IS over time in peripheral blood of T04228 (D). Clones with >1% frequency are depicted by individual boxes and colored if they appear at >1 time point. Clones <1% are grouped in to a single large gray box at the top of each bar. Shannon diversity index of clones in peripheral blood of T04228 (E).

456. Beta-Deliverin Treatment Enhances Lentiviral Vector Transduction of Human Hematopoietic Long-Term Repopulating Cells in NSG Mice

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Lentiviral vectors (LVs) are used to deliver genes to CD34$^+$ hematopoietic stem and progenitor cells (HSPCs) for treatment of a variety of hematologic disorders. However, the patient therapeutic benefit is dependent upon efficient LV gene transfer to hematopoietic stem cells (HSC). Achieving clinically relevant transduction levels in HSCs can require high amounts of LV, as well as extended *ex vivo* culture. LV production is costly, thus a possible impediment for clinical protocols, while *ex vivo* exposure to cytokines for prolonged periods may lower HSC engraftment. Methodology to increase HSC transduction while lowering the amounts of LV utilized is desirable for clinical translation. We previously reported that a small molecule, β-deliverin, improves LV HSPC transduction efficiency by enhancing LV fusion within endosomes. This resulted in ~2-fold increase in EGFP levels in cultured LV transduced HSPCs when compared to vehicle treatment. β-deliverin enhances transduction in cord (CB) and mobilized peripheral blood (PB) derived HSPCs, and non-human primate bone marrow (BM) HSPCs. We now report that β-deliverin enhances LV transduction of human long-term repopulating cells in NSG mice. In two independent studies, n=8 β-deliverin and n=8 vehicle control mice per study, mice were transplanted with β-deliverin treated or LV vehicle transduced (MOI 10 or 25) CB HSPCs. In one representative study, PB samples at 6 weeks from the β-deliverin mice demonstrated increased EGFP marking, x±S.D.=30%±16%, as compared to controls, x±S.D.=12%±4%; at 22 weeks EGFP marking in the β-deliverin mice remained 2-fold higher, x±S.D.=36%±19%, compared to controls, x±S.D.=15%±20%. β-deliverin treatment resulted in stable, increased EGFP levels with less variability over time as compared to controls. Total human cell numbers among individual mice in all groups were similar. High-throughput retrovirus integration site (RIS) analyses were performed on BM, spleen, and PB obtained from all humanized NSG mice. BM RIS analysis at 22 weeks post-transplant revealed abundant clones (defined as >20% of a single clone) in 10 of 16 mice transplanted with β-deliverin-treated cells, while only 1 of 16 mice receiving DMSO-treated cells exhibited clonal dominance. The total number of unique integration sites (IS) identified was ~50%-higher in mice receiving DMSO-treated cells. RIS analysis, however, showed no effect of β-deliverin-treatment on the global LV integration profile—including integration in or near oncogenes and transcription start sites—and dominant clones detected were not correlated with LV IS in known oncogenic genome regions. Moreover, the distribution and levels of human lineages in all tissues was unchanged by β-deliverin treatment, while gene marking was consistent with tissue EGFP marking. Analysis of unique RIS revealed preferred genomic regions for LV insertion for β-deliverin treated cells,

some of which were associated with highly abundant clones identified in the mice. Our results are consistent with β-deliverin treatment enhancing LV transduction of human long-term repopulating cells in NSG mice as evidenced by stable EGFP marking and no overt skewing of human lineages or levels. RIS analysis revealed heightened clonal marking, with no evidence of known oncogene insertion sites. We will discuss the implications of our studies in regards to enhanced HSC LV transduction within the current knowledge of hematopoietic clonal outgrowth post-HSPC xenotransplantation.

457. Evidence for the In Vivo Safety of Insulated Foamy Viral Vectors

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Retroviral vector mediated stem cell gene therapy is a promising approach for the treatment of hematopoietic disorders. However, genotoxic side effects from integrated vector proviruses are a significant concern for the use of retroviral vectors in the clinic. Insulated foamy viral (FV) vectors are potentially safer retroviral vectors for hematopoietic stem cell gene therapy. We evaluated two newly identified human insulators, A1 and A2, for use in FV vectors. These insulators had moderate insulating capacity and higher titers than previously developed insulated FV vectors. The A1 insulated FV vector was chosen for comparison with the previously described 650cHS4 insulated FV vector in human cord blood CD34$^+$ repopulating cells in an immunodeficient mouse model. To maximize the effects of the insulators on the safety of FV vectors, FV vectors containing a highly genotoxic spleen focus forming virus (SFFV) promoter were used to elicit differences in genotoxicity. In vivo, the A1 insulated FV vector showed an approximate 50% reduction in clonal dominance compared to either the 650cHS4 insulated or control FV vectors **(Figure 1)**, although the transduction efficiency of the A1 insulated vector was higher. Also, the A1 insulated FV vector had significantly reduced integrations within 50 kbp of TSS and within genes, as well as reduced integrations within 50 kbp of TSS of proto-oncogenes compared to either the 650cHS4 insulated or control FV vectors **(Table 1)**. This data suggests that the A1 insulated FV vector is promising for future pre-clinical and clinical studies.

Figure 1 Contribution of the top ten captured integrants. The average percent of the top ten integrants as determined by span is shown. *p<0.005. Error bars represent standard deviation. Abbreviations: FVSGW control vector, FVSGW-650cHS4-R 650cHS4 insulated, FVSGW-A1-F A1-insulated.

Table 1. Distribution of unique FV integration sites in transduced SRCs

Week		n	Total Integrations		Integrations in or near known proto-oncogenes	
			Within genes	Within 50 kb of TSS	Within	Within 50 kb of TSS
18	FV-control	384	44.5%	58.6%	8.6%	8.6%
	FV-650cHS4-R	258	48.4%	58.1%	10.1%	8.1%
	FV-A1-F	1 038	41.6%	54.4%[b]	8.1%	9.0%
27	FV-control	456	47.5%	62.8%	8.1%	10.1%
	FV-650cHS4-R	385	45.1%	59.3%	10.7%	11.0%
	FV-A1-F	913	38.6%[b]	54.4%[a]	8.1%	8.0%[c]

[a] significantly different than FV-control, p<0.001
[b] significantly different than FV-control, p<0.01
[c] significantly different than FV-control, p<0.05

458. Efficient KO of PD1 into Primary T Cells Using a New Non-Integrative Lentiviral Particle Expressing CRISPR/Cas9 System

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Gene editing by the CRISPR system shows great promises for gene therapy. Nevertheless, it must now face a number of challenges especially for the development of safe and efficient delivery tools for *in vivo*, as well as *ex vivo* gene editing. Cas9 and sgRNA delivery, mediated either by viral vectors (AAV- or Lentivirus-derived) or transfection protocols (chemical or by electroporation) have been largely and efficiently used but they bring major drawbacks incompatible with clinical applications. Indeed, viral vectors can display uncontrolled chromosomal integrations and transfection protocols are known to induce cell toxicity and/or phenotype modifications of the target cells. Moreover, the CRISPR technology entails a "hit-and-run" mechanism that only requires a transient expression of the nuclease complex. Therefore, achieving an efficient delivery into hard-to-transfect cells, such as T cells, remains challenging and the need for delivery tools that would allow efficient transfer on most cell types without causing any cell damages is essential for downstream therapeutic applications. Here, we present an innovative tool, named LentiFlash, allowing RNA delivery into target cells without any genomic scar. The RNA encapsidation is mediated *via* an RNA/protein interaction: the respective properties of the MS2 bacteriophage and the lentiviral vectors have been combined to build a non-integrative packaging system in which the wild type HIV packaging sequence is replaced by the MS2 stem-loop repeats and the MS2 coat protein is inserted into the Nucleocapsid. This new vector breaks with all existing systems, as the resulting lentiparticle is able to deliver non-viral coding or non-coding RNA, at high efficiency, into the cytoplasm of any cell type. Transduction of a large range of cells, from immortalized cells to delicate primary cells, such as T cells and hematopoietic stem cells, with LentiFlash shows an efficient, fast and transient expression of proteins and RNA, with no cell phenotype modification. In particular, LentiFlash particles were successfully used to deliver Cas9, alone or in association with an sgRNA not only targeting a reporter gene into immortalized cells, but outstandingly

knocking-out the PD-1 gene into primary human T lymphocytes. This new RNA delivery system is an efficient and safe tool for the delivery of CRISPR editing machinery in most cell types without affecting cell viability and phenotype. The transient, RNA-based mechanism of LentiFlash vector, preventing the risk of integration, associated with its ability to utilize lentiviral production platforms already validated in clinical settings, make it a promising tool for CRISPR therapeutic applications. As a matter of fact, beyond gene editing efficiency, safety of delivery tools is a major concern that should be addressed to move forward with CRISPR clinical development.

459. Complete Transduction of Spinal Cord Motor Neurons by Intra-Uterine Delivery of Integration-Deficient Lentiviral Vectors

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Integration-deficient lentiviral vectors (IDLVs) are promising gene delivery tools that retain the high transduction efficiency of standard lentiviral vectors, yet fail to integrate as proviruses and are instead converted into episomal circles. These episomes are metabolically stable and support expression of transgenes in quiescent cells, exhibiting a decreased risk of insertional mutagenesis. We have embarked on an extensive study to compare the transduction efficiency of IDLVs pseudotyped with different envelopes (vesicular stomatitis, Rabies, Mokola and Ross River viral envelopes) and self-complementary adeno-associated viral vectors, serotype-9 (scAAV-9) in spinal cord tissues after injection of mouse embryos (E16). Our results indicate that IDLVs can completely transduce motor neurons (MNs) regardless of the envelope pseudotype while scAAV9 mediates gene delivery to ~ 40% of spinal cord motor neurons with other non-neuronal cells also been transduced. Long-term expression studies revealed stable gene expression at 7 months post-injection. Taken together, the results of this study indicate that IDLVs may be efficient tools for cord transduction in therapeutic strategies such as for treatment of inherited early childhood neurodegenerative diseases.

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460. VISPA2: Faster and Extended Version of the Vector Integration Site Parallel Analysis Tool

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The analysis of the genomic distribution of viral vector genomic integration sites (IS) has a key role in gene therapy applications, since it allows the assessment of both the safety and the efficacy of the treatment, fostering in the meantime studies about the basic aspects of hematopoiesis and stem cell biology (Aiuti et al., *Science* 2013; Biffi et al., *Science* 2013). Vector integration sites are identified by high-throughput sequencing of PCR products containing the DNA junction between cellular and integrated proviral genome (Schmidt et al., *Nature Methods* 2007). Mapping the cellular genomic portion to the reference genome allows to accurately locate IS on the genome.We developed VISPA2 (Vector Integration Site Parallel Analysis, version 2), an improved version of VISPA (Calabria et al., *Genome Medicine* 2014), optimized in time-space consuming and in IS identification. The bioinformatics pipeline consists of several sequential steps, from pre-processing of raw sequencing reads to the creation of the final list of ISs with genomic context annotation. The major improvements concern:

(1) The use of the paired-end read aligner BWA-MEM, not just an end-to-end alignment, but local (portions of the read/contig) or chimeric alignments. (2) A more robust and accurate filter of low quality reads (developed in Python/Bash), custom quality filter specific for different kind of fragments (from Linear Amplification-Mediated or Sonicated Linker-Mediated - PCR). (3) Repetitive elements annotation (developed in Bash), after the reference genome alignment, taking account of the discarded reads from BWA-MEM (generally the 35% of the total reads) with RepeatMasker. (4) An innovative MATE and CIGAR filter (developed in Python) to remove reads with low mapping quality. (5) A new heuristic algorithm to correct wobbling of IS position effect caused by sequence/mapping errors in multiple sequencing reads. (6) A new intuitive, clear and simple web user interface (developed using Java and JavaScript) allowing a user-friendly analysis and annotation of integration site data (http://openserver.itb.cnr.it/vispa).

Moreover, VISPA2 performances showed increases of 2X in terms of memory, 6X in HD space and 7X time, in test datasets, compared to the old version.We measured VISPA2 precision (1.0) and recall (0.97) both on simulated and cell line experimental data and compared its results with other available tools (Mavric, SeqMap, QuickMap, VISPA) showing improved performances.In conclusion, VISPA2, that allows the analysis of large amounts of sequencing data (as in Big Data and Next Generation Sequencing era) such as those generated in gene therapy clinical trials and preclinical studies, is a step forward on integration site studies.

461. Development of Borna Disease Virus Vector for Differentiation of Human iPSCs into Skeletal Muscle Cells

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Background Borna disease virus (BDV) is a non-segmented, negative strand RNA virus that causes persistent intranuclear infection without causing cytopathic effect in various vertebrates. BDV is unique among the other *Mononegavirales* in that the viral replication takes place inside the nucleus of an infected cell. We have previously developed an RNA based vector system based on BDV, that can express a transgene from an extra-transcription cassette inserted between the viral phosphoprotein (P) and matrix (M) genes. BDV vector has been successfully used to transduce human stem cells, including mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs). In this study, to further explore the potential feasibility of using BDV vector for iPSC modification for gene therapy and regenerative medicine applications, we examined the ability of BDV vector to transduce various iPS cell lines derived from different cell types. In addition, to assess whether BDV vector can be used as a tool to induce differentiation of iPSCs into a particular cell type, we developed BDV vector armed with MyoD1, and induced differentiation of iPSCs into myocytes. Methods Seven iPS cell lines (201B7, 409B2, IISH1i-BM1, iPS11, BYS0110, BYS0112, and BXS0116) were transduced with BDV p/m-GFP vector. The level of transduction was determined at 3 days and 3 months post-transduction. To generate BDV vector for myogenic differentiation, MyoD was inserted into BDV p/m-GFP vector to generate BDV p/m-MyoD-GFP. Recombinant virus was obtained by transfecting 293T cells with BDV p/m-MyoD-GFP vector together with helper plasmids, followed by co-culturing with Vero cells. To induce myogenic differentiation, iPS cell lines 201B7 and 409B2 were transduced with BDV p/m-MyoD-GFP vector and cultured in skeletal muscle differentiation medium. The efficiency of myogenic differentiation was monitored by immunofluorescence analysis using muscle heavy chain (MHC) antibody. Results and Conclusion All iPS cell lines transduced with BDV p/m GFP vector became positive for GFP expression at 3 days post-infection. Furthermore, these cells remained GFP positive at 3 months post-transduction, indicating that BDV vector can efficiently transduce iPSCs derived from different cell types for a long period of time. 201B7 and 409B2 iPSCs transduced with BDV p/m-MyoD-GFP vector differentiated into MHC positive myocytes by 10 days post-transduction. Moreover, myocytes generated using BDV p/m-MyoD-GFP vector developed into multinucleated myofibers after incubation in myogenic differentiation medium. Our data further supports BDV vector as a promising vector system for efficiently transducing various types of iPSCs. These findings also support the future feasibility of using BDV p/m MyoD-GFP vector for conducting drug screening or disease modelling of skeletal muscle diseases.

462. Lentiviral Vector Integration Site Distribution in a Rodent Model of Parkinson's Disease

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With the progressive ageing of the population, the prevalence of Parkinson's disease (PD) and other neurodegenerative disorders is increasing. Gene therapy approaches are under consideration as treatments for PD, and lentiviral vectors are gaining increasing attention. In this work, a biosafety analysis of integrating lentiviral vectors encoding *eGFP* was performed using the 6-hydroxydopamine (6-OHDA) rat model of PD. Vectors were administered by stereotaxic injection into the striatum of both 6-OHDA lesioned and non-lesioned rats. Integration sites (IS) were analyzed by linear amplification-mediated polymerase chain reaction in combination with next generation pyrosequencing. The data from both lesioned and normal rodent brains showed similar integration frequencies, close to random integration profiles concerning chromosomal distribution and intragenic IS frequency, and no preferential targeting of cancer genes. A bias against integration at the proximal 5' region of the transcription start site (TSS) was encountered. Differences between lesioned and normal brain tissue were only observed regarding relative integration frequencies in various types of repetitive elements. Overall, the 6-OHDA lesion had no major impact on the lentivector integration pattern. These results support the use of lentiviral vectors for the treatment of PD by gene therapy.

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C.C.B, M.G.S and M.B. contributed equally to this work.

463. Identification of a Novel Viral Glycoprotein for Efficient Lentiviral Vector Pseudotyping and T Cell Transduction

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BACKGROUND: The vesicular stomatitis indiana virus glycoprotein (VSVG) is a widely used envelope for pseudotyping lentiviral vectors (LVs) due to its high titer, stability and broad tropism. However, the high fusogenic potential of VSVG prohibits the development of stable packaging cell lines that constitutively express VSVG. In addition, it is not clear if this envelope is optimal for transducing human T cells. Therefore, alternative envelope proteins were explored.**METHODS:** In this study, we generated 16 plasmids encoding different envelopes from the Vesiculovirus *genus* and compared their properties to VSVG. **RESULTS:** In a transient transfection system, 6 out of 16 glycoproteins

generated LV titers of over 10^7 transducing units/ml. Among the 6 glycoproteins, the envelope from maraba virus improved LVs titer by 80% compared to VSVG. Maraba-pseudotyped LVs were also efficiently concentrated by ultrafiltration. We observed that maraba-LVs are 60% more efficient at transducing primary human T cells across multiple donors when titer was normalized. Furthermore, 293T cells stably expressing the maraba envelope produced significantly higher LV titers than VSVG expressing cells. Maraba-LVs also showed improved infectivity of cyno T cells when compared to VSVG-LVs. **CONCLUSSIONS:** These studies illustrate that maraba-pseudotyped LVs offer a great alternative to VSVG-LVs for gene transfer applications, especially in engineered T cell therapy field. The generation of a stable maraba envelope expressing cell line can facilitate the development of a stable LV packaging cell line which will allow the production of large-scale, high titer clinical grade vectors at lower cost.

464. Comparison of Two Lentiviral Two-Step Transcriptional Amplification Systems Using GAL4FF or GAL4vp16 Transactivators for Ex Vivo Regional Gene Therapy in Bone Repair

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Introduction: Ex vivo regional gene therapy using a two-step transcriptional amplification (TSTA) lentiviral vector (LV) expressing Bone morphogenetic protein 2 (BMP2) under the control of the GAL4vp16 transactivator has been used successfully to induce bone repair in vivo. However, GAL4vp16 has been shown to be cytotoxic when overexpressed. Thus a different GAL4 transcription activator (namely GAL4FF), that has displayed less toxicity in prior studies, was investigated. The aim of the study was to develop a LV-TSTA-BMP2 vector that would enhance gene expression with minimal cytotoxicity for use in ex vivo gene therapy strategies using human MSCs to augment osteogenesis.

Methods: The TSTA system requires 2 different LV vectors, namely the transactivator and the transgene expression vector. Two transactivator vectors were constructed using the RhMLV promoter and either GAL4FF or GAL4vp16. The transgene expression vectors, encoding the G5 promoter and BMP2 or Green fluorescent protein (GFP), were then constructed (LV-G5-BMP2 or LV-G5-GFP). **(Fig 1)** MSCs, isolated from bone marrow (BMSC) and adipose tissue (ASC), were transduced with GAL4FF TSTA or GAL4vp16 TSTA, expressing BMP or GFP. Transduction with TSTA-GFP and analysis via flow cytometry were done to determine transduction efficiency and mean fluorescence intensity (MFI) 2 and 7 days post transduction. Transduction with LV-TSTA-BMP2 was then used to compare in vitro cell viability and BMP production, as quantified by ELISA, between the two TSTA-BMP2 vectors at 2, 7, 14 and 28 days.

Results: FACS analysis of GFP-transduced MSCs confirmed successful transduction with both TSTA-GFP vectors at MOIs of 1, 25 and 50. There was a trend towards higher MFI at all MOIs in GAL4FF+GFP versus the GAL4vp16+GFP treated MSCs at 7 days. **(Table 1)** ELISA results confirmed abundant BMP2 production over 4 weeks, with the peak BMP expression detected at 1 week for both the GAL4FF+BMP2 and GAL4vp16+BMP2 vectors (BMSC: 737±546ng vs 138±3ng and ASC: 925±1153ng vs 365±376ng respectively, $p<0.05$). **(Fig 2A)** GAL4FF was superior with respect to BMP2 production at 1, 2 and 4 weeks and was associated with a higher cell viability at all time points compared to GAL4vp16. **(Fig 2B)**

Conclusion: To our knowledge, this is the first report of GAL4FF mediated BMP2 production by human MSCs. Compared to the standard GAL4vp16 TSTA vector, the GAL4FF vector was associated with lower cytotoxicity and higher in vitro gene expression in both BMSC and ASC. Further experiments are needed to assess whether the superiority of GAL4FF with regards to overall BMP2 production in vitro translates to more robust bone formation in vivo.

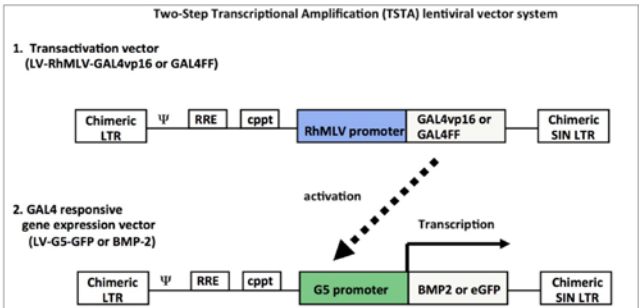

Figure 1. Structure of the lentiviral two-step transcriptional amplification (TSTA) system used to overexpress BMP-2 or GFP under the control of GAL4vp16 or GAL4FF transactivators. LTR, long terminal repeat; Ψ, packaging signal; RRE, Rev-responsible element; cPPt, central polyprine tract; SIN, self inactivating.

Transduction efficiency and MFI in ASC and BMSC transduced with TSTA-GFP (GAL4FF or GAL4vp16)

GAL4FF		ASC		BMSC	
		GAL4vp16	GAL4FF	GAL4vp16	
%GFP+	MOI=1	15 ± 5.2	16 ± 3.9	31.9 ± 10.9	42.8 ± 17.3
	MOI=25	65.4 ± 10.8	57.15 ± 7.4	95 ± 3.8	92.2 ± 1.5
	MOI=50	71.5 ± 12.7	63.65 ± 9.15	93.9 ± 3	91.3 ±1.6
MFI	MOI=1	8,666 ± 475	8,207 ± 291	7,130 ± 3,694	6,022 ± 2,533
	MOI=25	24,651 ± 2,246	22,587 ± 1,848	34,278 ± 4,124	31,189 ± 354
	MOI=50	29,840 ± 5,010	19,067 ± 8,462	38,624 ± 9,835	30,986 ± 3,918

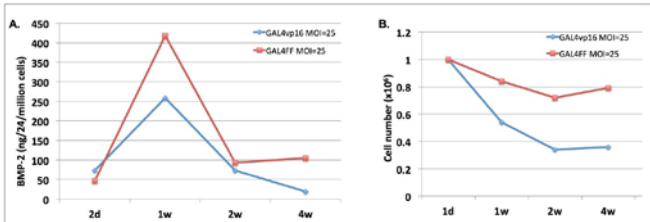

Figure 2. Bone marrow stem cells transduced with LV-TSTA-BMP-2 (GAL4FF+BMP-2 or GAL4vp16+BMP-2). **A.** BMP-2 production 2 days, 1 week, 2 weeks and 4 weeks post-transduction at MOI=25. The GAL4FF+BMP-2 transduced human cells produced more BMP-2 at 1, 2 and 4 weeks compared to GAL4vp16+BMP-2 cells. Results are given as ng of BMP-2 per million cells per 24h. **B.** GAL4FF+BMP-2 was associated with higher cell viability at all time points. Similar results were seen in ASC (data not shown).

465. Lentiviral Vectors Expressing a Hepatitis C Virus NS5A Motif Inhibit TCR-Mediated Activation of Human T Cells *In Vitro*

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Introduction: Viral vectors mediated gene-transfer based therapies have a promising future to treat many human diseases; however, host immune responses to viral vectors and its components limit the efficiency of gene transfer and the persistence of therapeutic genes. Thus, novel strategies to design viral vectors with lower immunogenicity are critical for the advancement of gene-transfer based therapies. Among RNA viruses, hepatitis C virus (HCV) is unique in its ability to persist in humans. HCV has evolved to modulate host immune response by multiple mechanisms. HCV NS5A protein inhibits Lck, a key enzyme required for induction of host T cell response. Specifically, the low complexity sequence (LCS-II) motif within NS5A is required for inhibition of Lck function. Here, we generated lentiviral vectors (LVs) expressing LCS-II and characterized its effect on human T cell receptor (TCR) signaling pathways. **Methods**: Two different LVs were generated to express the HCV NS5A (LCS-II) and the GFP transgene. GFP expression and inhibition of T cell function were compared between LVs containing the murine stem cell virus (MSCV) promoter without an internal polyadenylation signal (polyA, M-LV) and LVs with EF1α promoter and an internal polyA (E-LV). Primary human T cells transduced with engineered LVs were stimulated with anti-CD3/CD28 or PMA/Ionomycin to activate proximal or distal TCR signaling pathways. T cell activation was measured by assessing IL-2 release by ELISA, NS5A effect on TCR signaling was assessed by immunoblots and LVs transduction was measured by flow cytometry. **Results:** HCV NS5A LCS-II expression was significantly lower in human T cells transduced with M-LV compared to E-LV. Low level expression of LCS-II did not inhibit proximal or distal TCR signaling pathways. LCS-II expression was improved by incorporation of an internal polyA signal; however, consistent with previous studies, it resulted in a ten-fold reduction in LV functional titers. NS5A LCS-II did not interfere with GFP transgene expression in human T cells. Importantly, primary human T cells transduced with E-LV had a significant reduction in proximal TCR signaling compared to M-LV or control LV that does not express LCS-II. The E-LV mediated inhibition of T cell function was rescued following PMA/Ionomycin mediated activation. **Conclusions:** These data demonstrate that HCV NS5A LCS-II is sufficient to inhibit TCR signaling, and lentiviral vectors expressing LCS-II reduce TCR signaling *in vitro* without reducing transgene expression. Since LCS-II expressing LVs specifically inhibit proximal TCR signaling, incorporating LCS-II motif in the design of LVs may be an effective strategy to reduce vector immunogenicity *in vivo*. **Acknowledgements**: This work was supported by the Intramural Research Program of the CBER, FDA. This project was supported in part by Dr. Alan Baer's appointment to the Research Participation Program at CBER administered by the Oak Ridge Institute for Science and Education through US Department of Education and FDA.

466. A Smartphone Application for Quantitative Titration of Lentiviral Vectors

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Lentiviruses are extremely versatile viral delivery tools being used in a growing number of applications ranging from basic research to clinical applications. For nearly all these applications it is essential to accurately determine the infectious titer of the vector stock, as it contributes to the transduction efficiency as well as the final integrated copy number within the transduced cells. However, titration protocols are labor and time intensive, with timelines ranging from approximately 3 hours to 2 weeks. Here we present a smartphone application for the analysis of a lateral flow assay specific for lentiviral p24 protein that can provide infectious unit values (IFU/ml) in 10 minutes. The application functions on both iOS and Android devices. The assay consists of 2 steps: addition of lentiviral supernatant to the lateral flow cassette followed by imaging of the developed bands with a smartphone camera. The application compares the band intensity of the sample to a pre-loaded, lot-specific standard curve and produces a value that can be used to normalize virus stocks in a manner similar to using an ELISA titration assay. In addition, a reference virus with a known IFU/ml titer can be used to generate an IFU/ml titer for subsequent unknown samples. Using the software to analyze vectors made with several 3rd and 4th generation packaging systems, we obtained accurate titer values across a broad range of dilutions demonstrating R^2 values of 0.99 and coefficients of variation of less than 30% when compared to GFP titers on HT1080 cells. Taken together, this quantitative, inexpensive and highly convenient titration technology can decrease lentiviral vector titration time to approximately 10 minutes, reduce labor and material requirements, and expedite transduction experiments.

467. Lentivirus Production in Stirred-Tank and Packed-Bed Basket Bioreactor Systems: A Comparison

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Background: As gene therapy research progresses from basic studies into pre-clinical and clinical phases, there is a growing demand for large volumes of concentrated lentiviral (LV) vector, and accordingly, the means to produce such quantities. Unlike other viral vectors, lentiviruses are difficult to produce using stable cell lines, therefore transient transfection of adherent cells is conventionally used, and this method has proven challenging to up-scale. In this study, two bioreactor systems were evaluated as potential approaches for up-scaling LV production.

Methods: The New Brunswick CelliGen BLU controller and BioBLU® single-use vessels were used for both trials. For the stirred-tank

system, suspension HEK 293FT cells were expanded in FreeStyle™ 293 expression media to seed the 1.4 L bioreactor culture. At a density of ~ 1 x 10^6 cells/mL the culture was transfected with second generation LV plasmids using polyethylenimine (PEI), and 48 hours later the viral supernatant was harvested. The 3.75 L packed-bed basket was seeded with adherent HEK 293T cells in DMEM/10%FCS. At a cell density of ~ 3 x 10^4 cells/cm^2 the culture was transfected and 8 hours later the media was changed to OptiPRO™ SFM. 48 hours post-transfection the supernatant was harvested and processed using anion-exchange chromatography and ultracentrifugation. LV titres were quantified using a LacZ assay.

Results: The titre of the unconcentrated LV supernatant from the suspension stirred-tank trial was 2 x 10^4 TU/mL, while in comparison, the adherent packed-bed supernatant titre was 3 x 10^6 TU/mL. When concentrated the titre of the packed-bed preparation (resuspended in a final volume of 1 mL) was 5 x 10^9 TU/mL.

Conclusions: Lentiviral vector can be produced in both stirred-tank and packed-bed basket bioreactor systems. In this study, however, the adherent packed-bed system had a substantially higher production capacity. While further optimisation is necessary to ensure the capabilities of the two systems are maximised, both approaches show potential for large-scale LV production.

468. Scalable Suspension Lentiviral Production System for Cell and Gene Therapy Application

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Scalability of lentiviral vector production faces a big challenge to meet the demands for generating new T cell therapies CAR-T - TCR and high throughput drug screening by using lentiviral carried CRISPR/gRNA library technology. Both require a scalable lentiviral production system. We have developed a new lentiviral system to produce vectors in a serum free suspension platform which can be scaled up to bioreactors and wavebags system or scaled down to 96- deep well plates to produce LVVs at very high titers. Our system employs a newly developed propriety set of GMP reagents, special culture media, suspension cells, transfection reagent and enhancers. This system is able to produce >0.5-1E+08 (TU/mL) functional titer with un-concentrated LVVs in 2L production format, >2-3E+08 (TU/mL) in 96-deep well production format. In this report, we will demonstrate the robustness of using this system in large bioreactors and high throughput to fulfill the gene therapy and pharmaceutical industrial needs.

Synthetic/Molecular Conjugates and Physical Methods for Delivery of Gene Therapeutics I

469. Structure Activity Relationship of PEG-Peptide Scavenger Receptor Inhibitors

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The rapid capture and degradation of viral and non-viral gene delivery nanoparticles by scavenger receptors (SR) on Kupffer cells and fenestrated endothelial cells in the liver results in a decreased half life of all nanoparticles in an *in vivo* system, as well as a lower percentage of the dose available for delivery to the target organ. Current attempts to achieve SR inhibition involves co-administering high molecular weight, polyanionic inhibitors. Polyinosinic acid (Poly-I), the most widely used and potent inhibitor, competes for SR binding and successfully inhibits the uptake and metabolism of viral gene delivery nanoparticles in the liver. However, Poly-I also activates the immune system, resulting in toxicity in mice, making Poly-I clinically unacceptable. We have discovered peptide based SR inhibitors that block SR uptake of DNA nanoparticles and improves their metabolic half-life by forming protein nanoparticles in the blood (1). These Poly (ethylene) glycol (PEG) polylysine peptides potently inhibit SR and allow DNA to transfect hepatocytes up to 12 hours after administration in mice (2). Radio-iodinated PEG-peptides were used to study the pharmacokinetics and biodistribution to understand the structural properties which influence transfection competency and potency. We hypothesize that the *in vivo* potency and activity is influenced by the stability of protein nanoparticles formed in the blood. Peptides which are able to form stable nanoparticles are able to effectively block liver uptake of DNA nanoparticles by saturating SRs on Kupffer cells in the liver. The results of this study provide a framework for the design and synthesis of future PEG-peptide scavenger receptor inhibitors, as well as an assay to quickly determine the activity of each inhibitor.

1. Khargharia S, Baumhover NJ, Crowley ST, Duskey J, Rice KG. The uptake mechanism of PEGylated DNA polyplexes by the liver influences gene expression**. Gene Therapy**. (2014) 12, 1021-1028.

2. Baumhover NJ, Duskey JT, Khargharia S, White CW, Crowley ST, Allen RJ, Rice KG. Structure-Activity Relationship of PEGylated Polylysine Peptides as Scavenger Receptor Inhibitors for Non-Viral Gene Delivery. **Molecular Pharmaceutics**. (2015) 12, 4321-4328.

470. Optimizing Cardiac Delivery of Modified mRNA

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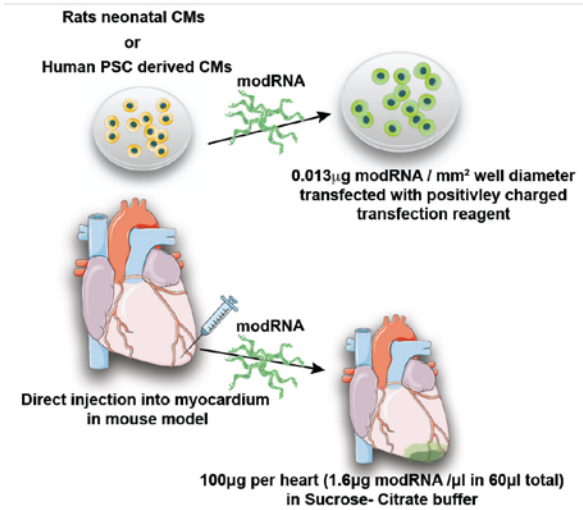
Abstract: Background: Modified mRNA (modRNA) is a new technology in the field of somatic gene transfer that has been used for delivery of genes into different tissues, including the heart. Our group and others have shown that modRNA injection into the heart results in robust expression of specific genes that lead to changes in cardiac function. However the optimal composition of the modRNA and the reagents necessary to achieve optimal expression in the heart have not been specified. In this study, we aimed to elucidate those parameters by testing different nucleotide modifications and doses of modRNA, and various transfection reagents both *in vitro* and *in vivo* in cardiac cells and tissue. Methods and Results: Using Luciferase (Luc) mRNA with or without nucleotide modifications we found that 100% replacement of Uridine by N1-Methylpseudouridine-5'-Triphosphate (1-mψU) results in lower immunogenicity, higher RNAse resistance and stronger translation compared to unmodified mRNA *in vitro* and *in vivo*. We show that transfection of modRNA in neonatal cardiac rat or human pluripotent stem cells (hPSC)-derived cardiomyocytes CMs require positively charged transfection reagents, however, in vivo direct injection into the myocardium, yields the highest transfection efficiency when delivered naked in sucrose- citrate buffer. Our results indicate that optimal cardiac delivery of modRNA (with 1-mψU modification) is achieved using 0.013µg modRNA /mm²/ ~500 CMs transfected with positively charged transfection reagent in vitro and 100µg per heart (1.6µg modRNA /µl in 60µl total) sucrose citrate buffer in vivo. Conclusions: We have optimized the conditions for cardiac delivery of modRNA in vitro and in vivo. Using the described methods and conditions will allow successful gene delivery using modRNA in various models of cardiovascular diseases where transient expression is required.



471. Efficient Vector-Free Engineering of MSC with Retention of Cell Viability and Differentiation Potential

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Genetic engineering of MSC while retaining cell function remains challenging, particularly for non-viral delivery methods which to date have been largely inefficient and hampered by toxicity. We have developed a novel vector-free method that uses reversible permeabilization to achieve rapid intracellular delivery of cargos with varying compositions, properties and sizes. A permeabilizing delivery solution was developed that contains a low level of ethanol as the permeabilizing agent. Reversal of cell permeabilization is achieved by temporally and volumetrically controlling the contact of the target cells with this solution. Cells are seeded in conventional multi-well plates. Following removal of the supernatant, the cargo is mixed with the delivery solution and applied directly to the cells using an atomizer. After a short incubation period, permeabilization is halted by incubating the cells in a PBS solution that dilutes the ethanol and is non-toxic to the permeabilized cells. Normal culture medium is then added. The procedure lasts less than 5 min. We have demonstrated that delivery occurs by diffusion directly into the cytoplasm in an endocytic-independent manner.

We evaluated the ability this method to achieve genetic engineering of primary human bone marrow-derived MSC using preassembled CRISPR/Cas9 protein-gRNA ribonucleoproteins (RNPs) and GFP mRNA respectively. Cell viability and differentiation capacity was also assessed. A comparison with electroporation was also carried out.

When Cas9 RNPs were delivered to MSC using this method, edit efficiencies of 49.5 % were achieved compared with 27.7 % using electroporation and cell viability levels were 76.0 % compared with 68.9 % using electroporation. When cells were induced to differentiate towards an adipocyte lineage following RNP delivery, there was no difference between untreated cells and cells treated with this new method. However, differentiation was inhibited in cells that were treated by electroporation. For GFP mRNA delivery, expression levels of 39.3 % were achieved.

In summary, this delivery method provides an efficient means of engineering MSC while retaining cell viability and differentiation potential. The method is gentle yet highly reproducible, compatible with high throughput and automated cell-based assays and has the potential to enable a broad range of applications.

472. DNA Vaccination of Subcutaneous Adipose Tissue Using Electroporation

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Introduction

DNA vaccines are clinically effective when delivered using electroporation (EP). However, the typical intramuscular EP route is associated with transient pain and muscle contraction, and requires invasive electrode needles to be inserted at the treatment site. Here, we

have developed a technique that specifically targets electroporation to subcutaneous adipocytes, in a bid to noninvasively transfect adipocytes *in vivo*, with the aim of reducing the invasiveness of DNA vaccination.

Methods

Using a simulation-driven approach, the electric field distribution within adipose tissue was modeled for different electrode designs. Using an optimized design, subcutaneous fat pads of guinea pigs were transfected by performing a subcutaneous injection of plasmid DNA followed by noninvasive electroporation, and reporter construct expression was monitored for 60 days. To demonstrate immunogenicity of this technique, guinea pigs were vaccinated with a flu construct, using adipose-EP at administered with different EP parameters and injection techniques.

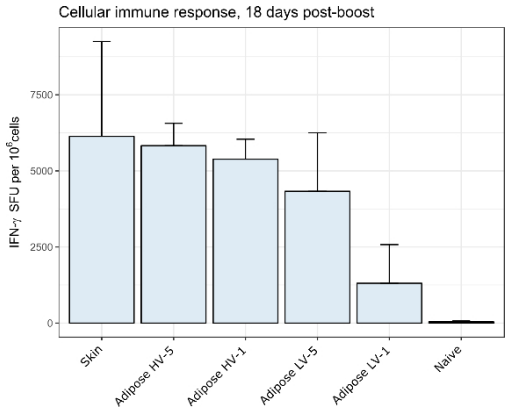
Results

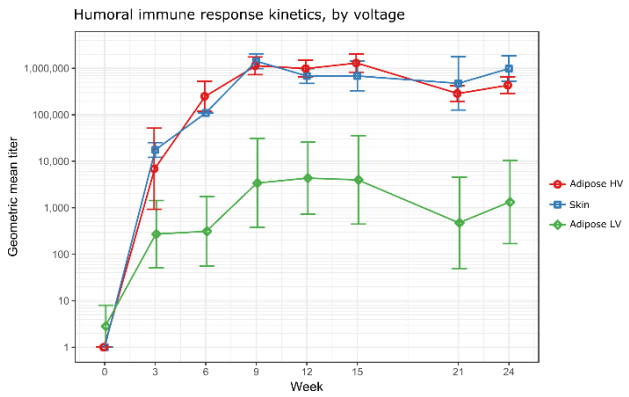
A noninvasive electrode design was shown to provide superior electric field distribution to standard needle electrodes. This design was capable of transfected large numbers of adipocytes within the treated area, and transfected adipocytes expressed encoded protein for at least 60 days. The optimized adipose-EP technique generated humoral and cellular immunity comparable to skin-EP, and the magnitude and kinetics of this immune response were dependent on DNA distribution as well as EP voltage (Figure 1).

Conclusions

Large numbers of adipocytes can be transfected in vivo using an electroporation device designed to concentrate the electric field at the site of subcutaneous DNA injection. These transfected adipocytes are capable of mounting a rapid, strong immune responses even with low doses of DNA typically used for intradermal treatments. Because of the potential tolerability and usability advantages, adipose-EP is a promising new modality for DNA vaccination.

Figure 1. Immunogenicity of adipose-EP vaccination using plasmid encoding flu antigen, in guinea pigs. Treatments were performed at 0, 3, 6, and 21 weeks. Left: kinetics of humoral immune response. Right: T-cell response 18 days following the week 21 boost.





473. ARRDC1- and Histone-Enriched Exosomes Released from Starved Stem Cells Improve siRNA Transfer to Neurons

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Introduction: Starvation has been reported to enhance release of exosomes from cells. We hypothesize that starvation of cells may impact exosome biogenesis, exosome protein composition, and exosome trafficking properties to transfer small RNAs between cells. Methods: Bone marrow derived (Lonza) and umbilical cord Wharton's jelly derived (ATCC) mesenchymal stem cells were cultured in stem cell medium depleted from exosomes, or in RPMI without any supplement for serum starvation. Exosomes were purified from mesenchymal stem cells *via* differential ultracentrifugation. Purified exosomes were loaded with hydrophobically modified siRNA (hsiRNA) and applied to primary neurons in culture or directly infused into striata of mice. hsiRNA distribution and mRNA silencing were quantified by peptide nucleic acid (PNA) -hybridization and QuantiGene® assays, respectively. Protein content of exosomes was assessed by LC-MS/MS. Results: Exosome released from non-starved and starved cells both contained bona fide exosomal markers CD63, CD81, CD9, Alix, and Tsg101. Starved exosomes were enriched in both early endosome marker Rab5 and late endosome marker Rab7. When comparing the proteome of cells and their exosomes before and after starvation, we observed significant changes in four vesicle signaling pathways. (1) Upon starvation cells downregulated HOPS complex (homotypic fusion and vacuole protein sorting complex, required for degradation of multivesicular bodies) and upregulated recycling endosomal pathway. As a result, exosomes from starved cells were enriched in proteins in the recycling endosomal pathway: GLG1 from trans-Golgi network, Rab11, Rab34. (2) Proteasomal degradation, E2 and E3 ubiquitin ligases were depleted in starved cells, and released in exosomal fraction enriched in proteasomal proteins and ubiquitin. (3) Starved cells upregulated fetuin-A, a protein known to shuttle histones from the nucleus to exosomes. Accordingly, starved exosomes were highly enriched in

several histones. (4) Exosomes from starved cells exclusively contained ARRDC1 (arrestin domain containing 1), a ubiquitin ligase adaptor found in small vesicle budding from the plasma membrane. Exosomes from starved cells showed enhanced uptake and transferred siRNA 2-15 fold better to primary neurons than exosomes from non-starved cells. Conclusions: Upon starvation, mesenchymal stem cells upregulate recycling endosomal pathway and release exosomes enriched in histone possibly mediated by ARRDC1-dependent budding. These exosomes have enhanced intercellular trafficking ability.

474. Naturally Inspired Gene Carrier: Histone Modified Gold Nanoparticles

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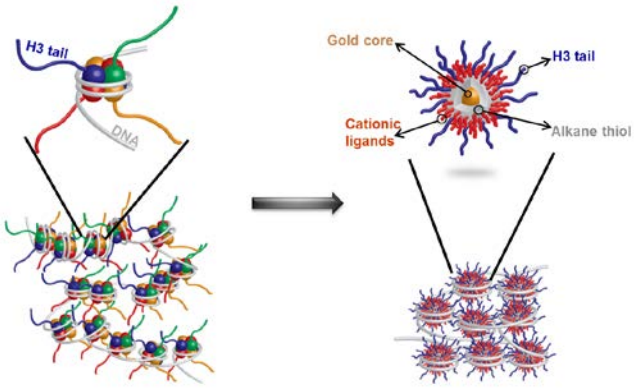
Histone proteins play important roles in nature's own mechanisms for gene packaging and transfer. Recent findings have expanded understanding of the histone tails in native gene regulatory control and subcellular trafficking. By incorporating histone tails within controllably assembled DNA vehicles (polyplexes), our lab has proved that polyplexes displaying modified histone 3 (H3) tails promote nuclear accumulation, DNA release, transcription, and enhanced transfection. Herein, we developed optimized multifunctional gold nanoparticle (AuNP) scaffolds decorated by histone motifs, which are supposed to induce efficient gene transfer. We hypothesize that polycationic AuNPs coupled to histone motifs will mimic the native presentation of these sequences on the histone octamer and create structures with the capacity to stably bind as well as controllably deliver plasmid DNA (pDNA) (see figure).



We prepared AuNPs with ~2nm cores based on the well-established Brust-Schiffrin two-phase method involving tetrachloroaurate reduction in the presence of 1-pentanethiol. Through Fmoc solid-phase synthesis procedures, short pentapeptides of poly-Lysine and histone peptides comprised of residues 1-25 of the H3 protein were successfully fabricated. These peptides were thiolated by conjugation with 11-mercaptoundecanoic acid. Subsequently, Murray place-exchange was employed to install various combinations of the thiolated peptides onto the AuNPs. Electron microscopy (EM) was used to analyze the core dimensions of the modified AuNPs. Thermogravimetric analysis (TGA) coupled with C/H/N/S elemental analysis was used to determine the average composition of multi-component ligands on the AuNP surface, and showed that H3-containing ligands were successfully installed at a variety of densities. To test the gene transfer potential of the histone-inspired AuNPs, stable nanoplexes were obtained through self-assembly of AuNPs and pDNA. The dimension and toxicity of the nanoplexes were easily controlled by fine-tuning the ligand composition of the AuNPs and the N/P ratio. Initial analysis of bioactivity showed that the H3-targeted AuNPs were capable of activating histone effector (HBO1), encouraging subsequent studies on AuNP engagement of histone pathways within cells for gene delivery applications.

475. Gut Localized PD-L1 Expression Using Dually-Derived Chitosan Gene Delivery to Treat Murine Colitis

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The intestinal immune system has evolved to maintain tolerance to a diverse community of commensal microbes. Loss of homeostasis within the mucosal environment can result in intestinal inflammation, leading to acute and chronic inflammatory bowel diseases (IBD). At the cellular level, loss of tolerance is induced by a decrease in regulatory T cell (Treg) number and function and an increase in pathogenic T cells. Immunoregulatory T cell costimulation by the programmed death (PD) 1 receptor (PD- 1) and its ligand PD ligand 1 (PD-L1) can provide a negative signal to inhibit T cell proliferation, mediate tolerance by promoting Treg development and function and prevent auto-inflammation. We hypothesize that modulation of PD-L1 levels in the gut *in vivo* provides a novel mechanism to control intestinal tolerance. We have developed a proprietary modified oligomeric chitosan, dually-derivitized chitosan (DD-chitosan), capable of packaging plasmid DNA into nanoparticles for *in vivo* delivery to mucosal tissues of the gastrointestinal tract. Codon optimized gene sequences of human membrane bound full-length PD-L1 or soluble PD-L1 protein (extracellular region fused to non-lytic human IgG1-Fc) were sub-cloned into the pVax expression plasmid. PD-L1 and PD-L1-Fc plasmids were formulated in DD-chitosan resulting in polyplex nanoparticles of defined size range. Protein expression and potency was confirmed following *in vitro* transfection. PD-L1-Fc was purified with Protein G affinity chromatography from the supernatant of transfected cells grown in serum free medium, quantified by ELISA and the purity confirmed by Coomassie stain. Full length membrane bound PD-L1 was quantified in cell lysate. *In vitro* potency of the PD-L1-Fc construct was assessed based on inhibition of T cell activation using T cells isolated from mouse splenocytes. Briefly, isolated T cells were activated with anti-CD3 with or without polyplex-derived PD-L1-Fc or recombinant PD-L1-Fc and activation was assessed by FSC/SSC shift using flow cytometry. *In vitro* potency of the membrane bound PD-L1 was assessed by PD-1 receptor binding assay using NIH/3T3 cells transfected to express PD-L1. NIH/3T3 cells expressing PD-L1 were incubated with or without PD-1 at various concentrations and flow cytometry was performed using anti-PD-L1 or anti-PD-1 fluorescent antibodies to assess binding. *In vivo* expression of PD-L1-Fc and PD-L1 was confirmed using RT-qPCR following intracolonic instillation of the polyplex to C57Bl/6 mice. To evaluate outcome in a disease model,

the murine T cell transfer model of colitis was used. Briefly, C57Bl/6 *Rag1*$^{-/-}$ mice received intraperitoneal injection of $CD4^+CD25^-CD45RB^{hi}$ naive T cells isolated from C57Bl/6 wild-type mice and 2 weeks post transfer mice were treated weekly with either PD-L1-Fc-PP, PD-L1-PP, pVax control-PP or vehicle buffer only. Significant effect on weight loss, survival and clinical signs was obtained with mice treated with PD-L1-Fc-PP and PD-L1-PP compared to mice treated with controls. Target engagement assessment showed a significant increase in FOXP3 expression by $CD4^+CD25^+FOXP3^+$ regulatory T cells in the mesenteric lymph node and splenocytes of mice treated with PD-L1-PP compared to plasmid control. Our data suggest that localized delivery of PD-L1-polyplex to the colon improves clinical manifestations of disease in a mouse model of IBD. Our findings provide a foundation for exploring the therapeutic effect of local delivery of immune checkpoint proteins to treat IBD using our DD-chitosan platform for gene delivery to the gut.

476. Enhanced Plasmid DNA Delivery from Photo-Triggered Stealth Liposomes

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For in vitro studies or in vivo applications, the delivery of nucleic acids including plasmid DNA (pDNA), single stranded DNA (ssDNA) or small interfering RNA (siRNA) is of major interest for gene delivery and therapy. Compared to viral vectors, nonviral delivery methods have gained tremendous attention due to their biocompatibility and less immunogenicity. Among the various nonviral vectors, cationic liposomes have attracted significant interests as a gene/drug delivery vehicle. Compared to traditional liposomes, stealth liposomes, characterized by modification of polyethylene glycol (PEG) on liposomal surface, have unique properties such as their enhanced stability, minimal recognition and clearance by the immune system and extended blood-circulation time. The polymer polyethylenimine (PEI) consists of secondary amine groups, which can enhance the binding of negatively charged DNA molecules to PEI to form PEI/DNA complexes. Additionally, PEI also can facilitate endosomal escape due to its extensive buffering capacity. However, the high toxicity of PEI largely limited its application as a promising DNA vector. By coating the polypolexes (PEI/pDNA complexes) with PEGylated liposomes, the formed lipopolyplexes (liposome/PEI/DNA complexes) will be able to reduce the cytotoxicity and enhance the cellular uptake, providing an efficient gene delivery platform.In this study we developed a facile stealth liposomal platform where PEI/pDNA complexes were loaded inside the middle cavity of a liposome and a photosensitive molecule was encapsulated inside a liposomal bilayer. A photosensitiser clinically used in photodynamic therapy, verteporfin, was chosen as photosensitive molecules. It can be triggered to generate the reactive oxygen species (ROS) under light illumination at a wavelength of 690 nm, destabilizing the membrane of a liposomal bilayer and the endo/lysosomes and resulting in the enhanced intracellular release of the loaded pDNA/PEI complexes. The light-triggering performance of these liposomes were demonstrated by the release of pDNA encoding green fluorescent protein (GFP) after red light illumination in cancer cells, HCT 116 and MCF7. Cancer cells which have taken up liposomes were exposed to 690 nm light illumination (15 mw/cm^2) at different time points (1 min, 5 min and 10 min). The subsequent endo/lysosomal escape of pDNA was documented based on quantitative analysis of colocalization between fluorescently labelled pDNA and endo-lysosomes. In addition, enhanced signals from GFP expression were observed with the time of photo irradiation, indicating an improved release of pDNA from light-triggered liposomes. In summary, our light-responsive PEGylated liposome delivery strategy was able to achieve enhanced endo/lysosomal escape of pDNA and GFP expression efficacy in cancer cells. Such liposomes would be able to achieve spatiotemporal control of gene release, potentially offering a non-viral gene delivery platform for efficient gene therapy. Therapeutic agents (eg. siRNAs and microRNAs) or gene editing systems (eg. CRISPR/Cas) would be able to be delivered and released in a more controllable way by taking advantage of such liposome delivery platforms in combination with light.

477. Effective Retroviral Transduction of Primary T Cells and Hematopoietic Stem Cells Using the Soluble Transduction Enhancer: Vectofusin-1

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Modification of target cells with retroviral vectors often requires the presence of a transduction enhancing reagent. Polycationic reagents induce aggregation of vector particles and aid binding to target cells via electrostatic interactions, while bridging molecules, such as recombinant fibronectin, interact with both vector particle and cell membrane. These methods can also be combined with centrifugation to further enhance transduction performance.

Transduction performance is highly dependent upon the pseudotype used and the receptor availability on the target cell. For example, VSVG-LV transduce primary T cells very effectively at low multiplicity of infection (MOI=1), while HSC often require 100-fold more vector. To overcome restrictions in viral vector entry to HSC, alternative pseudotypes have been developed, which also show reduced toxicity during production. LV pseudotyped with certain of these envelope proteins (e.g. RD114, GALV, BaEV) require an enhancement reagent to effectively bind and enter HSC and other cells such as T cells, while MV-LV pseudotyped with measles HF glycoproteins achieve good transduction rates also in the absence of enhancers.

We have assessed Vectofusin-1®, a histidine-rich, cationic amphipathic peptide, as an alternative transduction enhancer to modify both primary T cells and HSC. Vectofusin-1 is a short peptide of 26 amino acids which can easily be synthesized to high purity for clinical use. Unlike recombinant fibronectin, it is a soluble reagent which does not have to be precoated on cell culture surfaces, which makes automation of transduction processes for future clinical application less cumbersome.

CD34+ HSC were magnetically isolated from cord blood, pre-stimulated with a cytokine cocktail overnight and modified with LV at an MOI=10. 80% of HSC were modified using BaEV-LV in the presence of Vectofusin-1, while less than 5% of cells were transduced in the absence of the reagent. In contrast, MV-LV achieved almost 70% transduction in the absence of the enhancer and near to 100% gene modification when Vectofusin-1 was used.

We also compared the transduction performance of gammaretroviral vectors in the presence of Vectofusin-1 and recombinant fibronectin on primary human T cells that had been magnetically enriched from peripheral blood. After 2 days of activation with TransAct Reagent, the CD4+CD8+ T cells were transduced with GALV or RD114 pseudotyped gammaretroviral vectors encoding GFP at an MOI=1 or 2 respectively. Vectofusin-1 showed a comparable transduction performance to recombinant fibronectin (RD114, 35-45% GFP+ T cells; GALV, >60% GFP+ T cells). T cells modified with an ErbB2 chimeric antigen receptor (CAR) were fully functional.

We recently implemented a highly automated manufacturing process to generate LV-modified T cells based on the CliniMACS Prodigy® Platform, the T Cell Transduction (TCT) Process. We have further developed our process to enable gammaretroviral transduction in the presence of Vectofusin-1 within this single-use closed system

478. Synergistic Antitumor Effect Mediated by a Paclitaxel-Conjugated Polymeric Micelle-Coated Oncolytic Adenovirus

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Combination treatment consisting of oncolytic adenovirus (Ad) and paclitaxel (PTX) is a promising strategy to achieve synergistic antitumor effect. However, a co-administration approach is subject to inherent limitations due to the poor solubility of PTX and chemoresistance of tumor cells. In order to overcome these limitations, an oncolytic Ad expressing a p53 variant (oAd-vp53) that is resistant to p53 inactivation in the tumor microenvironment was complexed with PEGylated and PTX-conjugated polymeric micelle (APP). This approach generated an oAd-vp53/APP complex (176.4 nm in diameter) that could concurrently deliver both oncolytic Ad and the nanoparticulate drug APP to tumors. APP-complexed replication-incompetent Ad (dAd/APP) exhibited 12-fold higher transduction efficiency than naked dAd in coxsackie adenovirus receptor (CAR)-negative cancer cells. This increased efficiency was attributed to more efficient cellular internalization mediated by charge interactions between APP and anionic cell membranes. Furthermore, oAd-vp53/APP elicited synergistically higher cancer cell killing than naked oAd-vp53, APP, or oAd-vp53 in combination with PTX (oAd-vp53+PTX); this synergistic effect was shown to be due to superior induction of apoptosis and viral replication. Importantly, oAd-vp53/APP induced more potent and synergistic antitumor effect through both local and systemic administration by enhancing replication of oncolytic Ad and induction of apoptosis in tumor tissue. Further, the APP coating on the surface of Ad markedly attenuated the host immune response against Ad and decreased hepatic sequestration, resulting in minimal hepatotoxicity and a good safety profile. These attributes enabled oAd-vp53/APP to elicit potent antitumor effect over multiple treatment cycles. Altogether, we demonstrate that concurrent delivery of oncolytic Ad and APP as a single nanocomplex is a promising strategy for achieving synergistic antitumor effect.

479. A Poly-Functional Gene Delivery System for Cystic Fibrosis

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Objective: Cystic Fibrosis (CF) gene therapy is the only therapeutic strategy independent of mutations. Today, it is based on a safe and efficient delivery of *cftr* gene copy into pulmonary epithelial cells. Nevertheless, many extracellular obstacles such as the presence of bacteria can impair the efficiency of the gene transfer. Therefore, it is relevant to develop multi-functional systems combining a transfecting capacity and an antibacterial activity in order to eliminate the bacteria and thus facilitate the entry of the transgene into the target cells. A previous study has enabled us to identify a combination of a cationic lipid and a N-heterocyclic carbene-silver complexes that can be delivered by aerosol which has a transfecting capacity as well as a broad spectrum of antibacterial activity. The aim of this work was to develop new combinations of original silver salt and cationic lipids to increase the antibacterial effect and maintain the transfection efficiency while reducing the quantities of silver salt used. **Methods:** Firstly, the antibacterial activity against Gram + (*Staphylococcus aureus*) and Gram - (*Escherichia coli* and *Pseudomonas aeruginosa*) bacterial strains of each compound alone was evaluated in order to demonstrate a structure/activity relationship. Secondly, in contrast to the combination previously used (1/1molar ratio), the neo-synthesized N-heterocyclic carbene-silver complexes were combined with a cationic lipid in a 0.5/1 molar proportion of respectively. Thirdly, the transfecting activity and the cytotoxicity were then evaluated by direct deposition on bronchial epithelial cells. **Results:** All newly synthesized N-heterocyclic carbene-silver complexes showed antibacterial activity against Gram + and Gram - bacteria strains with minimal inhibitory concentrations (MIC) in the same scale (μM) of that of antibiotics. The antibacterial activities obtained with the neo-synthetic compounds were better against Gram + than those of the carbene-silver complexes previously used. When associated to a cationic lipid, the antibacterial activity was still obtained. The cytotoxicity induced from the new combinations was higher than the cytotoxicity observed with the previous carbene-silver complexes. The transfection efficiency by direct deposition of the new combinations was better than with the cationic lipid alone. **Discussion and Conclusion:** The addition of aliphatic chain on the silver salt was beneficial for the antibacterial activity. These new molecules have a very interesting antibacterial potential and could represent an alternative to antibiotics. In fact, preliminary tests allowed us to observe, with this type of compounds, the absence of resistance emergences of bacteria which are frequently isolated in

CF patients. Moreover, under infectious conditions (bacteria in the environment of epithelial cells), the combination of a cationic lipid with a carbene-silver complexes enables the transfer of the transgene into the target cells. For future research, it may be relevant to test new molar proportions in order to decrease the cytotoxicity and to evaluate these combinations following aerosolization.

480. An Apoptosis-Inducing Gene Approach Following Non-Viral Aerosolized Cationic Lipids: Application to Pulmonary Metastatic Bone Tumors

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Objective: Primary malignant bone tumors (osteosarcomas and Ewing's sarcomas) account for 6-10% of child and adolescent tumors. Approximately 15-30% of these tumors are already metastatic at the time of the diagnosis and the most frequent metastatic site is the lung (in 85% of cases). Despite an improvement in their management over the years, metastases still strongly undermine the disease prognosis, with a decrease in the 5-year free survival event from 70 to 30%. The main objective of this work is to evaluate the transfection efficiency of TRAIL by aerosol using synthetic gene delivery system and its ability to induce apoptosis. Clinical application would be the treatment of lung metastases of primary malignant bone tumors with aerosolized gene therapy. **Methods:** Several lipoplexes (cationic lipids/pDNA) have been tested, *in vitro*, on two different cell lines (KHOS, A673). The transfection activity was evaluated according to the expression of a luciferase gene reporter. The most efficient lipoplexes were then selected to aerosolize a plasmid encoding TRAIL. The results of the experiments were obtained by ELISA. In parallel, cells viability was measured. **Results:** The most efficient cationic lipid formulation was KLN25/DOPE/Cholesterol with a molar ratio of 1/1/1 respectively. It allowed a transfection efficiency of 2 log and a cell viability of more than 60% after aerosol. The transfection of TRAIL gene complexed with KLN25/DOPE/Chol with a molar ratio of 1/1/1 respectively leads to a relevant expression of the transgene in A673 cell lines. This accounted for a 40% reduction in cell viability. **Discussion and Conclusion:** This study shows that a transfection of primary malignant bone tumors on cell lines is possible by aerosol. The screening of synthetic cationic lipids must be continued in order to improve the transfection of the KHOS cells. This aerosol transfection technique can then be applied to a mouse model with pulmonary metastases of malignant primary bone tumors. Our goal will be to evaluate the efficiency of the transfection of TRAIL transgene in the treatment of pulmonary metastases of malignant primary bone tumors.

481. Graphene Oxide as a 2-D Platform for the Intracellular Delivery of siRNA

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The potential of graphene oxide (GO) as an intracellular carrier of small interfering RNA (siRNA) has so far been explored as a component of more complex delivery systems based on other materials, predominantly positively charged polymers, already used as gene delivery vectors for years but whose biocompatibility is far from ideal. In this study, we aimed to investigate whether bare GO could be utilized as a siRNA carrier on its own. The capacity of small GO flakes (lateral size < 1 μm, obtained by the modified Hummer's method) to deliver siRNA was interrogated in dividing and non-dividing primary cells, and in immortalized cell lines grown as monolayers and as three-dimensional (3D) spheroids. Its performance was compared to that of a benchmark lipid-based transfection agent (Lipofectamine®).

GO formed stable complexes with siRNA, in spite of unfavorable electrostatic interactions, and at GO:siRNA mass ratios (10:1, 20:1 and 50:1) that did not induce cytotoxicity. In addition, complexation was able to protect the nucleic acid from degradation, even at the lowest mass ratio tested and in the presence of high concentrations of nucleases that completely degraded free siRNA. Cellular internalization was confirmed by confocal microscopy using fluorescently-labeled siRNA. Importantly, siRNA was observed to travel further towards the core of 3D spheroids when complexed to GO, while the nucleic acid delivered by Lipofectamine® remained mainly in the outer cell layers. A PCR-based method allowed accurate quantification of the amount of effectively internalized siRNA and was used to compare the kinetics of siRNA intracellular delivery with both vectors. GO delivered siRNA rapidly (within 4 hours after transfection), however Lipofectamine® exhibited an entirely different pattern of intracellular transport and was able to sustain intracellular levels of the nucleic acid for longer, leading to more efficient levels of gene knock-down. We provide previously unreported evidence that GO (without any further functionalization with polycationic groups) is able to act as a flat, 2-dimensional siRNA carrier, able to transport short nucleic acids deeper and faster into avascular tissues. We conclude that it will be essential to further optimize such transport capabilities to achieve more sustained intracellular release, able to lead to effective knock-down.

Vector and Cell Engineering and Manufacturing

482. Development of a Monolithic Chromatography-Based Manufacturing Process for Clinical-Grade Viral Vectors

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Our current manufacturing protocol for clinical-grade Adenovirus-based vectors utilizes infection of suspension 293 cells grown with chemically defined media in disposable spinner flasks, and the virus purified by cesium chloride density gradient ultracentrifugation. Using this method, we have manufactured four batches of clinical-grade Adenovirus vectors that passed quality control testing on sterility, endotoxin and adventitious viruses. However, while this method can produce high quality, safe products that can be administered in clinical trials, the yield per batch has been limited as the ultracentrifugation method is not scalable. Furthermore, the use of 293 cells concomitantly results in the generation of replication-competent Adenoviruses (RCA). To improve our manufacturing process, we have recently tested the SF-BMAdR cell line (Gilbert *et al*, J. Virol. Methods 208, 2014), an A549-based cell line that expresses the Adenovirus E1 region. Two virus seed banks (8-liter scale) were manufactured using this cell line and both have been shown to be RCA-free (<1 RCA in 3 x 10^{10} Particles) by third party quality control testing. We have also acquired an NGC Quest Plus chromatography system (BioRad) and are currently establishing a virus purification method by anion exchange chromatography to support the transition from our current pilot scale manufacturing towards industrial scale manufacturing. In place of conventional resin-based columns, we focused our attention on the use of monolithic chromatography columns. The material inside these columns is a uniform, continuous network of large-sized pores and channels absent of any interparticle spacing. Thus, monolith columns obviate the usual mass transfer limitations associated with conventional resin packed columns and as such are ideally suited for the purification of large biomolecules such as virus particles. Polymethacrylate CIMmultus columns functionalized with quarternary ammonium groups (BIA Separations) were evaluated with Benzonase-treated and clarified adenovirus infected cell lysates. Gradient and step elution patterns were tested to maximize virus recovery and removal of impurities. Preliminary estimate of virus recovery from a 500 mL infected culture was 47%. Further work will involve large-scale purification (minimum 5-liter) as well as the characterization of contaminants (e.g. host cell proteins, host cell DNA) present in the purified virus fractions.

483. Production of Self-Complementary Vectors in AAV Producer Cell Lines

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Self-complementary AAV (scAAV) vectors bypass the requirement for second-strand DNA synthesis, enabling more rapid and efficient transduction. Recent clinical trials in Spinal Muscular Atrophy (SMA) and Hemophilia B have demonstrated the therapeutic potential of scAAV vectors. We describe herein a comparative analysis of the production of single-stranded (ssAAV) and self-complementary vectors (AAVrh10 seroptype) encoding the survival motor neuron (SMN) transgene in the scalable AAV producer cell line (PCL) platform. To generate AAV-SMN cell lines, HeLaS3 cells were transfected with a pre-PCL plasmid containing the AAV-SMN vector sequence, a puromycin resistance (puroR) selectable marker, the AAV2 *rep* and the AAVrh10 *cap* genes. High-producing scAAVrh10-SMN cell lines (vector yield of at least 1x1010 vg/mL) were infrequent, representing only approximately 0.1% of total candidates screened. In contrast, the prevalence of high-producing ssAAVrh10-SMN cell lines was higher (0.5-1.2%). Notably, however, the vector yields from high-producing scAAVrh10-SMN and ssAAVrh10-SMN lines were similar (7.5x104 to 1.2x105 vg/cell). The high-producing scAAV-SMN and ssAAV-SMN cell lines were compared with respect to biological (*e.g.* vector DNA replication and AAV gene expression) and vector quality (*e.g.* percent full capsids and non-vector DNA packaging) attributes. The only biological factor distinguishing the scAAVrh10-SMN and ssAAVrh10-SMN cell lines was a reduction (approximately 3-fold) in the number of total vector DNA copies in the scAAVrh10-SMN lines. A quality issue in the PCL-derived scAAVrh10-SMN vector was a high level of packaged non-vector sequences (*e.g.* puroR), ranging from 5-15% of vector DNA copies. Modification of the pre-PCL plasmid, including mutation of cryptic Rep binding sites in the puroR gene, resulted in a significant reduction of puroR DNA packaging. Experiments generating scAAV and ssAAV cell lines in the context of a different serotype (AAVDJ) suggest that the low success rate observed with the scAAVrh10-SMN cell lines might be serotype-dependent. In summary, though the overall frequency is less than ssAAV vectors, high-producing scAAVrh10-SMN cell lines have been isolated and an important attribute of vector quality, non-vector DNA packaging, has been addressed via plasmid engineering.

484. Novel Method for RNA Quantification and Exclusion from Plasmid DNA Vector Purification Process Stream

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Plasmid based DNA vaccines are emerging as a promising alternative to traditional vaccines due to several advantages. One of these advantages is faster production of plasmid DNA (pDNA) using *E.coli* host cells. The alkaline lysis process, described by Birnboim and Doly (1979), is often used for recovery of pDNA from *E.coli* cells. The lysis process releases unwanted molecules from the host cells including protein, RNA, endotoxin, and chromosomal DNA. The majority of chromosomal DNA and endotoxin are precipitated by the neutralization solution and removed by depth filtration of the resulting lysate. Host cell protein and small RNA molecules are generally removed by anion exchange chromatography (AEC) or tangential flow filtration (TFF) of the lysate. However, the remaining AEX or TFF process pool has a relatively high content (10 % to 50%) of large RNA molecules due to the physiochemical similarity of large RNA and pDNA. Rapid quantification of RNA impurity levels present in process intermediates is difficult due to the absorbance of both RNA and DNA molecules at A_{260}. A method for rapid RNA quantification method by nephelometric turbidity measurement upon selective precipitation (salting-out) of RNA from process intermediates using the chaotropic salts has been established. The direct correlation of Nephtometric Turbidity Units (NTU) to RNA concentration has established a useful tool in downstream purification processes, allowing more accurate determination of materials required, significant cost reduction in purification processes, and increased product quality by exclusion of RNA earlier in the process stream.

485. Scaling up the Lentiviral Vector Manufacturing Using iCELLis500 Clinical Scale Fixed-Bed Bioreactor

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Recognizing the need for efficient large-scale commercial adherent cell culture which is encountered in the field, Pall provides the ideal solution by way of the iCELLis® fixed-bed bioreactor. The single-use iCELLis bioreactor is designed for cultivation of anchorage dependent cells with surface areas of up to 500 m2. FinVector, part of Trizell Group, is a Gene and Cell therapy focused company based in Kuopio, Finland. Finvector has done pioneering work with iCELLis and developed clinical/commercial scale production processes for adenovirus type 5 and Adeno-associated viral vectors. Here we present a scalable and efficient method to produce lentiviral vectors with transient transfection of adherent human embryonic kidney 293T (HEK293T) cells in fixed-bed bioreactor. Several process parameters were firstly optimized at small scale, and for the first time in the World we also showed the scale up of lentiviral vector production into iCELLis500. Commercial scale upstream process development was followed by development of scalable downstream process. Virus containing medium was harvested by perfusion, concentrated and diafiltrated by tangential flow filtration and virus was purified using anion exchange chromatography. iCELLis500 offers a practical solution for viral vector clinical/commercial scale lentiviral vector manufacturing.

486. Suspension-Based Process for Lentiviral Vector Production Using a Stable Inducible Cell Line

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Scale-up is a major hurdle in the production of lentiviral vectors (LVV) for use in gene therapy as traditional methods for production involve transfection of adherent cells cultured in serum-containing medium. We have evaluated a suspension and serum-free adapted stable inducible LVV producer cell line (Broussau et al., Mol. Ther. 2008) for the production of 3rd generation LVVs carrying the GFP transgene.

Initial optimization was performed in shake flask cultures where we identified a media formulation that supported cell densities of greater than 5E6 cells/mL in batch culture. Cultures were induced by the addition of cumate and doxycycline to produce LVV. A feeding regime based on daily media exchanges yielded daily titres of greater than 1E7 TU/mL between day 2 to day 5 post-induction. This process was translated to a 1 L stirred tank reactor with media exchange via continuous perfusion. At this scale, we were also able to obtain daily harvested titres of greater than 1E7 TU/ml for 3-4 consecutive days. With further process development and optimization, this system shows promise in addressing future needs for large scale LVV production.

487. Large Scale Production, Purification and Concentration of Lentiviral Vectors Using Hyperstack Vessels, Anion Exchange Chromatography and Tangential Flow Filtration

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Lentiviral (LV) vectors are of particular interest to the field of gene therapy. LV offer a high rate of transduction into many different cell types, the ability to transduce non-dividing cells, stable gene integration, and a reduced risk of insertional mutagenesis. Yet clinical-scale production and purification of high-quality LV is difficult and costly. Herein we describe a methodology to scale-up production of high-titer LV using HYPERStack technology, a vessel consisting of many layers of gas permeable tissue culture treated growth surface in a fully closed, disposable system. Following LV packaging and harvest from the HYPERStacks we describe a method for LV purification by

way of Mustang Q anion-exchange membrane chromatography, and a method for LV diafiltration and concentration using Tangential Flow Filtration. Research labs around the world typically generate LV vector stocks by a plate-based culture strategy. We routinely generate LV stocks over 10^8 transduction units (TU) per ml using such methods. Using LV/eGFP we compared laboratory plate-based LV production to large-scale HYPER technology based LV production. Our results show that HYPERflask LV culture resulted in similar LV yield with significantly reduced incubator space required and labor hours invested. HYPERstack technology further reduces required labor, with minimal reduction in viral production yield per volume (0-15% reduction). Our results also indicate an increase in LV production when collection steps are completed in serum-free media (10-20% increase in LV production). Mustang Q anion-exchange membrane chromatography was used to purify and concentrate the LV. A 1000 fold volume reduction resulted in excellent viral recovery (75% of starting LV). Tangential flow filtration was used to further concentrate the LV down to 3 mL of volume, and to perform a diafiltration step into PBS. The result was not only diafilterred and purified LV, but two-fold the functional LV product that would be recovered from a similar volume laboratory plate-based approach. The HYPERStack LV packaging strategy, Mustang Q chromatography, and TFF purification regiment described here is a practical method for LV scale-up to early-stage clinical trial volumes using affordable disposable laboratory supplies. We will adapt these approaches as we generate GMP-grade vector for clinical trials in our Facility at the Medical College of Wisconsin.

488. Large-Scale Production of Lentiviral Vectors for Use in Therapeutic Applications

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Large-scale production of lentiviral vectors (LV) for therapeutic applications in gene therapy is necessary to achieve the full potential of this technology and meet clinical demand. Oxford BioMedica's delivery platform incorporates a minimal Lentiviral vector system, with key safety features such as SIN LTR and codon optimised gag/pol. Historically lentiviral vector manufacture has relied on an adherent process using serum containing media. We have now established a suspension cell production process up to 200 litre scale in serum free media. This has led to a significant increase in productivity due to better control of the growth environment of the cells. This not only improves the cost of goods but also allows the production of enough clinical material to treat diseases requiring large quantities of vector. Further enhancements to the process include an automated method for the generation of LV packaging and producer cell lines. This is a bespoke Automated Cell Screening System (ACSS) for the isolation of clones (>1000) by automated cloning by limiting dilution (LDC) utilising robotics. Furthermore, the ACSS can perform routine passage and high-throughput (HTP) clonal LV production and evaluation of productivity using various screening methods. This has resulted in the generation of LV producing cell lines that give equivalent or better titres than transient transfection processes. For most viral vector platforms, the maximal production titres are typically achieved with 'inert' transgenes such as GFP. However, expression of therapeutic transgenes during production can often adversely affect titres due to the biological effects on production cell metabolism and/or composition of the vector virion itself. Differential effects of transgenes can also variably alter the composition of crude harvest material, potentially requiring bespoke optimisation of downstream processing steps specific to each therapeutic vector. We have developed the novel 'Transgene Repression In vector Production' (TRiP) system for the production of both RNA- and DNA-based viral vectors that minimises transgene expression leading to recovery of production titres (at or close to maximal). This represents a major step towards development of transgene-independent viral vector manufacture. These advances in vector manufacture applied in an industrial scale process bring significant improvements in potency, purity, yield and efficiency which will set the benchmark for commercial manufacture of lentiviral vectors for therapeutic use.

489. Transmission Electron Microscopy Analysis of Accumulation and Aggregation of Adenoviral Structural Proteins During Vector Downstream Processing

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Adenoviruses are among the most commonly used vectors for gene therapy and large-scale manufacturing processes have been developed for the production of high-titer adenoviral vectors.

Not all adenoviral structural proteins are incorporated into virion particles during the production process and additional viral proteins are released if vector particles are broken during the process. These proteins, unless removed by the downstream purification steps, can aggregate or promote aggregation of vector particles.

This study explored the capability of transmission electron microscopy to detect viral structural proteins, their aggregates and broken viruses in samples from clinical grade adenovirus downstream processing. We used MiniTEM transmission electron microscope, which is capable of automated image acquisition and viral particle and debris counting. Automated operation of the system enabled us to save large number of images for subsequent analysis. In this study, automated, semi-automated and manual image analysis methods were used.

The development of algorithms and staining practices for particle analysis is an ongoing process, but our current results allow us to observe changes in adenovirus vector preparation particles numbers, homogeneity and purity during the downstream processing. We were also able to link microscopy results with the product aggregation tendency and provide useful information for process development purposes.

490. Process Characterization of Engineered Autologous Cell Therapies

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As engineered autologous cell therapy products move quickly through clinical development into commercialization, a well-defined, controlled, GMP-compliant manufacturing process is required. Process characterization is an exercise to define the manufacturing process and is a critical precursor to process validation. Due to the inherent donor-to-donor variability in both cell composition and quality that exists when working with patient cells, characterization of a robust and capable manufacturing process is challenging. In contrast to monoclonal antibody production and cell culture in general, industry knowledge for the production of engineered cell therapies such as anti-CD19 chimeric antigen receptor (CAR) T cells is not as developed. This gap provides opportunities for process optimization and characterization. Due to the promising clinical results of anti-CD19 CAR T cell therapies recently reported, development timelines require multiple activities to occur in parallel. Process characterization of autologous therapies is challenged by the limited availability of starting cell material from patients. As such, the use of surrogate cells and alternative approaches need to be considered. Here we present three approaches (a process risk assessment, clinical manufacturing data review, and healthy donor studies) that can be used to characterize the manufacturing process of anti-CD19 CAR T cells. The overall benefits and limitations of each approach are described. A common way to characterize a manufacturing process is to identify and classify process parameters through a process risk assessment. In a process risk assessment, the process parameters are identified and ranked by their risk to final product quality. This risk-based approach is recommended in the ICH Q8 guideline and is familiar to auditors and reviewers. Clinical manufacturing data review is another approach to process characterization and takes advantage of the abundance of manufacturing experience gained during clinical development of autologous products. This approach did reveal critical process parameters not previously identified and clarified the degree of process variability that can be observed when processing patient cells. Another approach is to study the manufacturing process in a controlled environment. For autologous cell therapy products, this requires the use of surrogate starting material such as healthy donor cells. By removing the donor-to-donor variability from the study design, the true impact of a raw material attribute or process parameter was studied. These three approaches, when used together, resulted in a robust, well-characterized, risk-driven definition of the manufacturing process.

491. Use of Dynamic Light Scattering to Optimize Scale-able Transfection Complex Formation for AAV Production in Serum-Free HEK293 Suspension Cultures

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Manufacturing of adeno-associated virus (AAV) for gene therapy applications is typically performed using either the baculovirus system in suspension insect cells or through triple transfection of adherent HEK293 mammalian cells. A primary advantage of the baculovirus system is the process control and scale-ability of suspension systems, while a primary advantage of the mammalian system is the relative ease of designing and sourcing plasmid DNA rather than baculovirus constructs. To further improve AAV production processes we sought to optimize mammalian conditions in suspension adapted 293-derived cells. To this end, we have manufactured a cGMP cell bank of HEK293 cells adapted to serum-free suspension culture and developed protocols for triple transfection in the bioreactor using low cost, easily constructed plasmid DNA and commercially available transfection reagent. The system enables rapid screening of drug candidates and translation into the clinic. Formation of the complex between transfection reagent and DNA is critical to achieving high transfection efficiency and subsequent AAV production.

Factors such as transfection reagent-to-DNA ratio, concentration of reagents during complex formation, ratios of the three plasmids, incubation time, and media used for both complex formation and cell culture are important and must be determined empirically. For transfections of large scale cultures, mixing and transfer methods must be significantly altered and become additional factors to explore and control. All these interacting factors, result in a very large design space. Because bioreactor experiments are expensive and time-consuming to conduct, it is necessary to narrow experimental conditions using simple, high through-put methods.

As transfection reagent is incubated with plasmid DNA, the particle size increases rapidly. Particles which are too small may not contain all three required plasmids, while particles which are too large do not enter the cell as efficiently. Instruments that measure particle size and distribution by dynamic light scattering (DLS) can be used to monitor the formation of the transfection complex. Identification of conditions that produce particles of the appropriate size then allows selection of appropriate ranges for critical factors, reducing the number of required experiments in bioreactors. Using the DLS method and quantification of viral genomes, we have identified conditions for reliable scale-up from spinner flasks to 50 L bioreactors.

492. Progress Towards a Scalable Manufacturing Platform for Recombinant Ancestral Adeno-Associated Virus Vectors

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Adeno-associated virus (AAV) is an emerging class of safe and potent viral vectors used for gene therapy in clinical trials. However, their broader application requires new advances in vector bioengineering and bioprocessing to overcome some of the current vector limitations. As a key challenge, pre-existing immunity (PEI) is observed in up to 70% of people who have prior exposure to wild-type AAV. PEI adversely impacts the delivery of AAV vector *in vivo* via neutralizing antibodies and other mechanisms, essentially excluding the majority of patients from AAV clinical trials and treatments. One of the most enticing potential solutions to this challenge is provided by a novel *in silico*-derived Ancestral AAV vector (Anc80) that shows favorable escape from PEI and high transduction efficiency for liver, muscle, and retina. Notwithstanding the remarkable potency of the vector, widespread clinical use of Anc80 will require robust and scalable manufacturing processes in which critical quality attributes can be maintained to ensure quality of the drug product and patient safety. We are establishing modern, best-in-class, robust, cGMP- compliant large-scale manufacturing platforms for Anc80 production. Here we will present a pilot study of upstream production of Anc80 vector using triple transfection method in suspension HEK293 cells. In the study, AAV2-GFP and/or AAV/Anc80-GFP productivity was assessed through clonal cell line establishment, cell culture media evaluation, and transfection reagent screening, including plasmid ratio and source. Future work is required to improve the productivity of the potent AAV vector through further process optimization.

493. Safety Assessment of the MACS GMP T Cell TransAct, a Robust and Potent Polyclonal T Cell Activation and Expansion Tool for Clinical Scale Manufacturing of Gene-Modified T Cells

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Automated manufacturing of gene-modified T cells for adoptive T cell therapy requires robust and reproducible processes using particularly materials and reagents which fulfill requirements for a safe application of cellular products. Aiming to streamline the safe and robust clinical manufacturing of gene-engineered T cells, we have developed a cGMP compliant stimulation reagent, the MACS GMP T Cell TransAct, which allows potent polyclonal T cell activation prior to gene-modification (lenti- and retroviral) without the need for feeder cells. Moreover, it can be be easily integrated into a closed manufacturing process. The MACS GMP T Cell TransAct is a polymeric nanomatrix conjugated to humanized CD3 and CD28 agonists. It is soluble, can be sterile filtered and is biodegradable for better usability. In contrast to the large beads previously used in the field, MACS GMP T Cell TransAct presents several advantages: "debeading" steps are not required resulting in simplification of the manufacturing process. As the reagent is soluble, there is no more critical debendence on the bead to cell surface density ratio which enables more reliable stimulation of the T cells within a large range of T cell density. However, using immunemodulating substances for the manufacturing of cellular products for e.g. advanced T cell therapies, a safety assessment of these reagents is essential to identify potential risks associated to their use. Here the safe use of the MACS GMP T Cell TransAct within the automated CliniMACS Prodigy platform using the TCT application software is demonstrate. In order to address potential risks, a series of in-vitro tests have been carried out: a dose-response curve (corresponding to the detection of the minimum anticipated biological effect level) was used to determine the safe application levels via determination of the dilution at which the TransAct is no longer capable of yielding T cell stimulation. Furthermore, the dilution factors of TransAct during a standard manufacturing run on the CliniMACS Prodigy were calculated. Based on these fold dilutions and correlating them with the inflection point of the dose response curve the product-associated risk during a TCT run was evaluated. In addition, highly sensitive in-vitro assays were carried out to verify whether potential traces of TransAct remaining in the supernatant of culture or bound TransAct at the surface of T cells is functionally active. At last, it was determined whether the CD28 agonist used in TransAct shows superagonistic behavior similar to the immunemodulating mAB TGN1412. In conclusion, it could be demonstrated that at dilutions over 100-fold of the initial working dilution, no activation of T cells was detectable. In correlation with the MACS GMP T Cell TransAct dilution during a standard manufacturing run, it can be stated that past day 6 the remaining TransAct is diluted below its lowest activation concentration. In addition, the supernatant of a T cell culture is not able to activate fresh T cells when taken after day 7 of the primary culture and activated T cells are not capable of cross-stimulating other T cells. Finally the CD28-TransAct nanomatrices do not show superagonstic behavior.

494. GMP Manufacturing of Lentiviral Vectors: Capacity and Scale Considerations

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The number of very exciting and high profile products based on gene and cell therapy has increased dramatically over the past few years. Consequently, advanced therapeutics now attract significant interest from the wider biotech/Pharma and investment communities. For the past 20 years, Oxford BioMedica (OXB) has been a pioneer in the development of products based on lentiviral vectors, with the company being responsible for several firsts in clinical studies based on these vectors. OXB is using this broad CMC, clinical and regulatory experience and know-how to facilitate product development based on lentiviral vector technology, both for company pipeline products and those of our strategic partners. The presentation will outline the different and parallel strategies being adopted by OXB to develop a

manufacturing capability to generate material with the suitable quality attributes, appropriate capacity and with acceptable cost of goods. This includes novel methods to prevent transgene overexpression interfering with vector particle production and purification and high-throughput methodologies for generating and screening producer cell lines. These developments will help to enable realisation of the full clinical benefit of these exciting and potentially curative products in patients. Developments in process improvement and scale-up will be discussed, as will the capacity planning strategy to provide security of GMP supply.

495. Industrializing the Hospital - A Fully Closed, Automated Method for Processing Hematopoietic Stem Cell Transplants

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Although there are very few cell therapies in commercial production, hematopoietic stem cell transplantation (HSCT) is regarded as an established procedure. Isolated from peripheral/cord blood or bone marrow, hematopoietic stem cells have the ability to repopulate the body's immune system after treatments such as chemotherapy. HSCTs are the standard of care for a variety of disease states with over 50,000 patients receiving transplantations annually[1]. This established treatment continues to grow 10-20% annually, operating under minimally manipulated conditions, regulated by FACT. Currently the majority of manufacturing is undertaken within hospitals by highly skilled operators with multiple manual, open manipulations and potential for critical deviations to occur. This, alongside the high level of manufacturing variation, has highlighted the need for both harmonization of the process and a move towards automated, closed processing. PCT, in collaboration with Invetech, have developed a fully closed, automated manufacturing platform that resolves the aforementioned problems and maintains the flexibility required to account for biological variation. By automating the process, the variability inflicted by manufacturing is vastly reduced, labor costs are reduced and the risk of error minimized. Closing the system minimizes the risk of contamination and lowers the facility costs. Such a system can be placed within the same hospital facility and bring these core advantages whilst reducing variability, labor and facility costs, improving patient care and permitting faster patient turnaround. The availability of a flexible platform to be placed within the hospital setting allows for expanding hospitals manufacturing capability to meet the HSCT product demand whilst ensuring consistent quality, scalability, sustainability and a reasonable cost of goods. The data presented in this study demonstrates the ability to attach a fresh apheresis unit, wash, volume reduce, formulate, and fill multiple bags ready for controlled rate freezing, all in a completely closed and automated manufacturing platform utilizing counter-flow centrifugation (CFC platform). The programmable CFC platform allows multiple unit operations to be conducted on one system, reducing the manual transfer stages, multiple disposable sets and reducing the capital expenditure required for multiple instrumentation. This also allows for multiple patient lots to be manufactured in the same room, with reduced operator oversight, heavily reducing the labor time and therefore allowing a sustainable, affordable scale out approach, necessary for patient-specific manufacturing. Through automation the skill level of operators can be reduced whilst reducing the risk of sterility issues. Hospitals can also bring all processing in-house, to simplify logistics and provide an improved patient experience. Figure 1 depicts the process flow moving from fresh apheresis through to a volume reduced, cryopreserved product. This has the potential to transform the current standard of HSCT and make these therapies accessible to all.

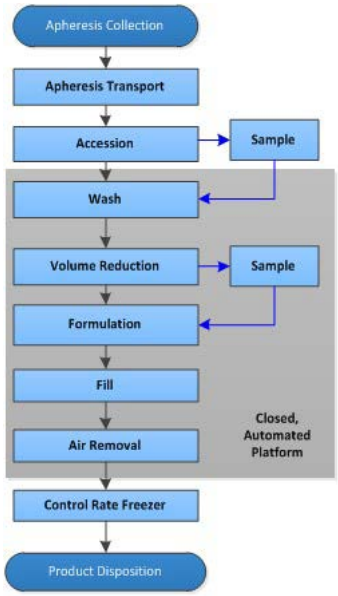

Figure 1: Process flow Diagram - Closed, automated HSCT process.

1 - Worldwide Network for Blood and Marrow Transplantation. Media Fact Sheet: 1 Million Blood Stem Cell Transplants Worldwide. WBMT. Available at January 30, 2013; Accessed: January 25, 2017

496. Cell or Virus Production within a Barrier Isolator Reduces Biosafety Risks

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Virus production poses increased risks to laboratory workers beyond those of routine laboratory work. Even with modern use of the biological safety cabinet (BSC), laboratory acquired infections (LAI) are still being reported from routine cell handling. No clear route of infection (needlestick, animal bite, ingestion or splash) was noticed in cases reported publicly in 2016. This makes inhalation of unseen particles the most likely route of infection. Airborne droplets are generated by routine open operations when a liquid film is broken, such as in uncapping tubes, or when energy is added to a solution, such as when pipetting, vortexing, or centrifuging solutions. When working in a BSC, people are exposed to airborne particles when their arms or other objects interrupt the BSC's laminar air flow. This introduces small particles into the room close to the operator's face. Larger particles can settle out of the processing space onto the worker's gloves and sleeves to be carried to the worker's face outside of the BSC. The Xvivo system barrier isolator provides a physical barrier between critical processing spaces and the operator. It can also actively remove particles from the internal atmosphere. The continuously recirculating atmosphere cleaner (CRAC) system recirculates the internal atmosphere through

HEPA filters. Using the null hypothesis that the CRAC system would make no difference in the persistence of 0.5 micron particles in the processing chamber, we tested three routine laboratory operations performed in virus production, pipetting, opening tubes, and centrifugation. Real-time particle monitoring showed that with the CRAC in operation, generated particles were rapidly cleared from the processing space. With the CRAC off, particles remained airborne for long periods of time. We concluded that the barrier isolator with the CRAC system in operation was able to rapidly eliminate particles generated by routine cell handling operations from the air. Along with the extra physical barrier between workers and the cell and virus production space provided by the barrier isolator, this type of active particle control can help prevent exposure of the operator to potentially infectious particles during virus production.

Presidential Symposium

497. CRISPR/Cas9-Mediated Targeting of a CAR into the TRAC Locus Enhances CAR T Cell-Mediated Tumor Rejection

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Chimeric antigen receptors (CARs) are synthetic receptors that redirect and reprogram T cells to mediate tumor rejection. The most successful CARs used to date are those targeting CD19, which offer the prospect of complete remissions in patients with chemorefractory/relapsed B cell malignancies. CARs are typically transduced into patient T cells using γ-retroviral or other randomly-integrating retroviral vectors (RVs), which may result in variegated CAR expression and transcriptional silencing. Recent advances in genome editing enable efficient sequence-specific modifications in human primary cells, including site-specific transgene integration into the CCR5 and AAVS1 loci in T lymphocytes. We have found that directing a CD19 CAR to the human T cell receptor (TCR) alpha chain (TRAC) locus not only results in efficient and uniform CAR expression in human peripheral blood T cells, but, remarkably, also enhances T cell potency, vastly outperforming that of conventionally generated CAR T cells in an Acute Lymphoblastic Leukemia (ALL) mouse model. We further show that CAR gene expression under the control of the TCR alpha promoter reduces tonic signaling, and allows effective regulation of CAR expression upon antigen encounter. In addition, these TRAC-CAR T cells show delayed T cell differentiation and exhaustion when stimulated multiple times. These findings further advance the proficiency of T cell engineering and highlight the potential of CRISPR/Cas9 genome editing to advance immunotherapies.

498. Safety and Efficacy of Human Embryonic Stem Cell Derived Oligodendrocyte Progenitor Cells (AST-OPC1) in Patients with Subacute Cervical Spinal Cord Injury

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AST-OPC1 is a cryopreserved population of cells derived from human embryonic stem cells which contains oligodendrocyte progenitor cells and other characterized cell types. AST-OPC1 has three potentially reparative functions including production of neurotrophic factors, stimulation of vascularization, and induction of remyelination of denuded axons. The initial safety of AST-OPC1 was evaluated in a phase 1 clinical trial that enrolled 5 patients with neurologically complete T3-T11 thoracic spinal cord injury (SCI). Based on favorable 5 year safety data, a phase 1/2a clinical trial (SCiStar) is underway to evaluate the safety and activity of escalating doses of AST-OPC1 in patients with severe C5-C7 SCI. The SCiStar trial is an open-label, single-arm trial testing doses of $2x10^6$, $1x10^7$ and 2 $x10^7$ AST-OPC1 cells in 5 cohorts of patients with sub-acute, C5-C7, motor complete (AIS-A or AIS-B) cervical SCI. AST-OPC1 is administered 14 to 30 days post-injury. Patients are followed by the International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) neurological exam and other assessments including MRI to determine safety and activity. Thirteen subjects have been dosed to date. Cohorts 1 (AIS-A, $2x10^6$ cells) and 2 (AIS-A, $1x10^7$ cells) are complete with 3 and 6 subjects, respectively. Enrollment in cohorts 3 (AIS-A, $2x10^7$ cells), 4 (AIS-B, $1x10^7$ cells) and 5 (AIS-B, $2x10^7$ cells) is progressing. The results to date from patients in cohorts 1 and 2 indicate no safety issues associated with the delivery of AST-OPC1 or the cells themselves. As further evidence of safety, no subjects in either cohort demonstrated decreased neurological function after administration of AST-OPC1. To date, all patients in cohorts 1 and 2 have had improved upper extremity motor function as assessed by upper extremity motor scores (UEMS) and their neurological level of motor function. The average UEMS improvement for the 3 patients in cohort 1 was 6.3 (range 3-10) points at 6 months, with each patient demonstrating one neurological level improvement in motor function. Early evidence of a dose response is emerging with patients receiving $1x10^7$ AST-OPC1 having greater motor recovery. The 5 patients in cohort 2 who have completed 6 months of follow-up, recovered an average 9.8 (range 6-16) motor points with continued improvement for the three subjects who have reached 9 months of follow-up. Likewise, 2 of the 5 patients that have been followed in cohort 2 for at least 180 days have achieved a 2 motor level neurological improvement. The motor function improvement in patients in cohort 2 compares favorably with a closely matched historical group of 62 traumatic spinal cohort injury patients derived from the EMSCI (European Multicenter Study about Spinal Cord Injury) database of over 3300 patients. Further enrollment and follow-up of patients continue. The data to date demonstrate the safety of

AST-OPC1 and provide encouraging early evidence of improvements in arm, hand and finger function in patients with C5-C7 complete cervical spinal cord injury.

499. Antisense Oligonucleotide-Mediated Dnm2 Knockdown Prevents and Reverts Myotubular Myopathy in Mice

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Centronuclear myopathies (CNM) are severe non-dystrophic muscle diseases characterized by muscle weakness and hypotrophic fibers with centralized nuclei. The most severe form, myotubular myopathy or X-linked CNM (XLCNM), is caused by myotubularin (*MTM1)* loss-of-function mutations, while the main autosomal dominant form is due to dynamin2 (*DNM2*) gain-of-function mutations. No specific therapy is available for patients. We previously showed that the myopathic *Mtm1* Knockout (*Mtm1*KO) mice over-express DNM2 and that genetic reduction of DNM2 in these mice, by crossing with *Dnm2*+/- heterozygote mice, prevents the development of the muscle phenotypes. This genetic proof-of-concept highlights epistasis between *Mtm1* and *Dnm2*, and validates the concept of cross-therapy where downregulation of a CNM gene rescues the loss of another CNM gene. However, translation to a therapeutic application requires a deliverable compound. Here we demonstrate that systemic delivery of the recently developed constrained ethyl (cEt) antisense oligonucleotides (ASO) into *Mtm1*KO mice at early phase of the disease reduces efficiently DNM2 protein level and prevents the myopathy progression while rescuing both lifespan and body weight, and correcting muscular mass, histology and force in a dose-dependent manner. Noteworthy, systemic injections of ASO into severely affected *Mtm1*KO mice reverted different myopathy signs after only 2 weeks and prolonged lifespan by normalizing DNM2 protein level. In conclusion, this data validates the efficacy of cEt ASOs for a non-dystrophic myopathy and demonstrate that normalization of DNM2 level through ASOs reverts and corrects CNM features after disease onset, providing an attractive therapeutic strategy that may be applied to patients with centronuclear myopathy.
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500. *In Vivo* Gene Silencing with *S. aureus* CRISPR-Cas9 Repressors

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CRISPR-Cas9 epigenetic repressors have demonstrated promise for silencing target gene expression efficiently and specifically *in vitro*. Adapting programmable transcriptional modulators for use *in vivo* would enable the study of gene regulation in complex organisms and the development of novel therapies to address aberrant gene regulation in disease. Recently, a Cas9 nuclease derived from *S. aureus* was described for delivery in adeno-associated viral vectors (AAV) and *in vivo* gene editing. We genetically fused a KRAB motif to *S. aureus* nuclease-null dCas9 (SadCas9) to create a RNA-guided repressor compatible with AAV gene delivery. To test the efficacy of SadCas9-KRAB *in vivo*, we targeted the *Pcsk9* gene in the liver of a wild-type mouse model. Strategies to silence *Pcsk9* are currently being explored clinically to lower harmful low-density lipoprotein (LDL) cholesterol and treat hypercholesterolemia. Intravenous delivery of a dual-vector AAV9 system expressing SadCas9-KRAB and a *Pcsk9*-targeting guide RNA (gRNA) resulted in a dramatic reduction in PCSK9 protein expression, with serum PCSK9 levels dropping to 18% of sham-treated control levels within 2 weeks of treatment and maintaining >75% reduction of serum PCSK9 through 6 weeks post-treatment compared to sham and SadCas9-KRAB only controls. We also observed significant Pcsk9 transcriptional silencing in the liver at 6 weeks post-treatment by qRT-PCR. Concomitant with *Pcsk9* silencing, we detected increased LDL receptor protein expression in the liver and >25% reduction in total cholesterol and LDL cholesterol levels in the serum compared to controls. We performed RNA-sequencing to investigate global effects of *Pcsk9* silencing and AAV-mediated dSaCas9-KRAB delivery on gene expression in the liver. Differential mRNA expression analyses showed increases in gene expression of immune regulators with AAV-mediated SadCas9-KRAB delivery (false discovery rate, FDR < 0.05). However, expression of hepatic genes and serum levels of alanine aminotransferase remained relatively stable compared to sham controls, suggesting that liver function is not compromised by AAV delivery or SadCas9-KRAB expression. Additionally, reductions in serum cholesterol are maintained through 16 weeks post-treatment, demonstrating that durable phenotypic effects are possible *in vivo* with RNA-guided transcriptional repression. In ongoing studies, we are investigating potential immune responses incited by AAV or SadCas9-KRAB expression and characterizing the specificity of *in vivo* CRISPR-Cas9 epigenome editing. Here, we target *Pcsk9* as a potential strategy to treat hypercholesterolemia, but this SadCas9-KRAB delivery system can be customized to silence any endogenous gene, enabling potent and stable gene repression in animal models and for therapeutic applications.

Evolution and Library-Based Engineering of AAV Vector Systems

501. Discovery of a Neurotropic Footprint That Enables AAV Transport Across the Blood-Brain Barrier

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Effective gene delivery to the central nervous system (CNS) by intravenously administered recombinant adeno-associated viral (rAAV) vectors requires crossing the blood-brain barrier (BBB). Consequently, in order to achieve therapeutic levels of transgene expression in the CNS, high vector doses (e.g., 1e14 vg/kg in the spinal muscular atrophy trial NCT02122952) are often required. In addition

to burden associated with scale up and costs, high vector doses have also been shown to cause undesirable side effects such as liver toxicity. In order to improve the specificity/efficiency of gene transfer to the CNS and lower the effective vector dose, a better understanding of the structural features that enable AAV capsids to penetrate the BBB is needed. To dissect structure-function correlates for crossing the BBB, we generated a combinatorial library of shuffled capsid genes using only two serotypes - AAV1, which does not traverse the vasculature and AAVrh.10, which is known to efficiently cross the BBB. Rather than evolve novel chimeric variants, we selected individual variants by computational, phylogenetic and structural analyses for further screening in mice. Following intravenous administration, we identified a subset of chimeric capsids capable of crossing the BBB and efficiently transducing the CNS. Structural modeling and sequence analyses further helped identify several key clusters of residues in AAVrh.10 implicated in the phenotype of crossing the BBB. Subsequently, we were able to further narrow down the size of this footprint through a rational approach, which was then functionally validated in vivo. In conclusion, we have identified a minimal footprint from AAVrh.10 which, when grafted onto other AAV strains, permits transport across the BBB and enables a more targeted CNS transduction profile compared to AAVrh.10. Furthermore, the resulting capsids are detargeted from the brain vasculature, liver and other peripheral tissues. The functional mapping of this novel neurotropic footprint provides a roadmap for engineering synthetic AAV capsids for efficient CNS gene transfer with an improved safety profile.

502. Bioinformatic Identification and Large-Scale In Vitro and In Vivo Characterization of Human-Derived Novel AAV2 and AAV2/3 Capsid Variants Reveals Vectors with New Biological Functions

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Recombinant adeno-associated viruses (rAAVs) have emerged as highly favorable gene delivery vectors for human gene therapy. Conceptually, probing the human population for AAV diversity, which is driven by selective pressure and/or immune evasion, has great potential for yielding clinically relevant vectors. In this study, we examined AAV proviral capsid sequence libraries isolated from a large panel of clinical biopsy samples, which include, brain, breast tissue, liver, lung, pancreas, prostate, stomach, and tumor tissues. To recover full-length proviral capsid sequences from AAV-positive tissues, we used single-molecule, real-time (SMRT) sequencing followed by bioinformatics filtering to remove sequences that may originate from library preparation or sequencing error. This novel and robust approach has revealed over 800 unique capsid variants that predominantly carry 10-20 amino acid variances from the prototypical AAV2 or AAV2/3 caspids.In order to characterize such a large number of novel candidate variants for high-packaging efficiencies during vector production and unique in vivo transduction profiles, we designed a two-part screening pipeline. At present, we have subjectively selected an initial 50 AAV2 and AAV2/3 variants to assess the robustness of our pipeline design. Preliminary characterization has revealed multiple AAV2 variants with remarkably higher vector packaging efficiencies than the prototypical AAV2 capsid and a subset of variants that exhibit the unique ability to efficiently transduce the submandibular region of mice after tail-vein delivery. Interestingly, the signature heparin-binding domain of the AAV2 capsid is altered in these novel AAV2 variants. The biology underlying such distinct vector properties is currently being investigated. On the other hand, all AAV2/3 hybrid capsids tested in vivo have thus far behaved more like AAV3B rather than AAV2, showing little transduction of any mouse tissues by tail-vein injections. Collectively, this study demonstrates that the use of SMRT-sequencing to interrogate AAV-host tissues as sources for viral diversity can potentiate the discovery of new AAV capsid sequences with distinct vector characteristics, and will pave the way for new rAAV-mediated gene therapy strategies. L.L., G.X., and P.W.L.T. are Co-first authors; Y.W. and G.G. are Co-corresponding authors.

503. Novel AAV Variants Isolated by Directed Evolution in Primate Display Enhanced Retinal Transduction Following Intravitreal Injection

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Directed evolution of AAV capsid libraries in mice has identified variants with enhanced transduction of mouse retina following intravitreal (Ivt) injection. In primate, however, the barriers to transduction are more formidable. To address this we developed a method to generate separate, sortable retinal cell populations in macaque (*M. fascicularis*) which enabled screening of a highly complex AAV2-based capsid library and identification of AAV variants capable of efficient retinal transduction following Ivt injection. In parallel, the same library was screened in mice. After 3 rounds of selection in mice and, separately, 2 rounds of selection in macaque, a subset of the most prevalent variants was characterized. Here, we evaluate their transduction profiles following Ivt injection in mice. We also determine whether the transduction efficiency of select variants could be increased by incorporation of rationally designed capsid mutations. After identification and isolation, capsid variants of interest were vectorized with a self-complementary AAV genome carrying the truncated CBA promoter driving mCherry (sc-smCBA-mCherry). Transduction was quantified *in vitro* using ocular cell lines. Vectors were Ivt injected into Nrl-GFP mice with constitutively fluorescent, sortable photoreceptors and transduction was evaluated 4 weeks later by fundoscopy and FACS. AAV2wt and AAV2(quadY-F+T-V) vectors were included in experiments as a basis for comparison. Sequence analysis of recovered

variants indicate motifs that promote interaction with the inner limiting membrane of the retina were highly selected. After 3 rounds of screening in mice, two heavily enriched variants were chosen for characterization. After 2 rounds of selection in macaque, the four most prevalent variants were also selected for characterization. Interestingly, there was overlap in the variants arising out of mouse and primate screenings at each respective stage. All of the characterized variants displayed improved transduction *in vitro* compared to AAV2wt. When evaluated for transduction following Ivt injection in mice, all variants, with one exception, displayed greater transduction than our previously most efficient capsid, AAV2(quadY-F+T-V). The addition of tyrosine to phenylalanine (Y-F) and/or threonine to valine (T-V) mutations onto select variants resulted in a substantial increase in retinal transduction. Incorporation of four proteosomal avoidance mutations onto the most enriched capsid variant in the second-round primate screen produced a variant that transduced >4.5-fold more rod photoreceptors following Ivt injection than AAV2(quadY-F+T-V). Lastly, after a third round of screening in macaque, several capsids were identified that displayed biased enrichment in either retinal ganglion cells or photoreceptors demonstrating the potential for isolation of capsids with cellular tropism. Taken together, we show that AAV capsids identified by screening in primate and mouse retina show enhanced transduction efficiencies both *in vitro* and following Ivt injection.

504. Structure-Guided Iterative Evolution of Antigenically Advanced AAV Variants for Therapeutic Gene Transfer

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The problem of pre-existing neutralizing antibodies (NAbs) against Adeno-associated viruses (AAV) poses a major and unresolved challenge for clinical gene therapy using recombinant AAV vectors. In order to enroll for ongoing clinical trials, patients must satisfy stringent exclusion criteria requiring very low to undetectable titers of anti-AAV NAbs. Here, we combine structural information from cryo-reconstruction of capsid-antibody complexes and antigenic footprint mapping with accelerated evolution to select antigenically advanced AAV variants. By iteratively engineering common antigenic motifs (CAMs) on the AAV capsid, we generate new, synthetic capsid surface topologies that are not present on ancestral, extant, or mosaic AAV strains. One variant, AAV-CAM130, derived from AAV serotype 1, mediates robust gene transfer to cardiac tissue as well as the brain. More importantly, CAM130 efficiently evades anti-AAV1 neutralizing sera obtained from pre-immunized mice and rhesus macaques. Further, the CAM130 variant displays robust escape from broadly neutralizing antibodies in naïve primate and human serum samples at dilution factors as high as 1:5, thereby eliminating exclusion criteria mandated by several ongoing clinical trials. The technology platform described herein can be applied to any AAV strain - natural isolate, or engineered variant. Thus, our approach yields antigenically advanced AAV vectors that can evade NAbs across multiple species and expands the cohort of patients eligible for gene therapy.

505. Directed Evolution of AAV Vectors Guided by Deep Sequencing Creates Variants That Bypass Structural Barriers in Canine Retina

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Introduction: Delivering AAV vectors across physical barriers to reach therapeutic cellular targets is an obstacle for the success of gene therapy and is therefore a major goal in the field. In the retina, several substantial physical barriers prevent the spread of viral particles, including the inner limiting membrane as well as layers of second and third order neurons. Naturally occurring serotypes of AAV are unable to overcome these hurdles to infect photoreceptors and RPE cells, the primary targets of retinal gene therapy. Therefore, we used *in vivo* directed evolution to create new AAV variants with the ability to overcome structural barriers and infect the outer retina. Deep sequencing was used to find best-performing variants, which were unidentifiable through traditional Sanger sequencing of a small sample size.

Methods: This work was performed in canines, a valuable preclinical model of inherited retinal degeneration, given their eye structure similarity to humans and previous use to validate vectors for human clinical trials. We show here that directed evolution resulted in the creation of new variants with the ability to efficiently infect the outer retina of this large animal eye following intravitreal injection. Following iterative selection of a library of 10^7 distinct capsid variant AAV vectors through intravitreal injection into the eye of a wildtype dog, AAV vectors were recovered from the outer retina. Isolated variants were then combined and re-injected, and following 5 rounds of selection, they converged to a pool containing the most successful variants. Convergence and variant performance was analyzed using deep sequencing of all rounds.

Results: Deep sequencing was used to characterize the process of directed evolution with unprecedented accuracy and to select the best performing variants. Importantly, this analysis revealed top-performing variants based on fold increase over their representation in the original library in addition to frequency in the final round of selection, which was discovered to be biased by initial overrepresentation. Twenty-one lead candidate variants were packaged with a cassette containing GFP fused to a barcode, pooled, and tested simultaneously in canine retina. Following injection of the GFP-barcode library, samples were recovered, and deep sequencing was used to quantify GFP by DNA and mRNA expression levels. Injection of evolved variants mediated high levels of reporter expression in photoreceptors and RPE, as well as other cell types. Interestingly, injections in rodents showed that dog-selected variants demonstrated species specificity. The top variant, which was designated K9#16 and had highly improved tropism for the outer retina compared to naturally-occurring serotypes, is being evaluated for therapeutic gene delivery in canine models of retinal degeneration.

Conclusions: These results show that *in vivo* directed evolution in canines, guided by deep sequencing, yielded new vectors for more efficient gene delivery to the outer retina. Furthermore, this work highlights the need for quantitative analysis of variant fitness, and supports the promise of *in vivo* directed evolution for the development of gene therapy vectors with new capabilities. Ongoing experiments are being performed to characterize the efficiency of these canine-derived vectors.

506. In Vivo Selection of a Computationally Designed SCHEMA AAV Library Yields a Novel Variant for Infection of Adult Neural Stem Cells in the Subventricular Zone

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Adult neural stem cells confer plasticity to the central nervous system and have neuroregenerative capabilities that may be harnessed to treat disease. Significant progress has been made in elucidating the molecular mechanisms that govern neural stem cell maintenance and neurogenesis in the subventricular zone (SVZ), the largest germinal niche in the adult mammalian brain. Our understanding of stem cell biology has improved, yet the ability to genetically manipulate endogenous stem cell populations *in situ* remains challenging due to inefficient vehicles for gene delivery. To address this need, we applied a Cre-dependent *in vivo* directed evolution and selection strategy to engineer an adeno-associated virus (AAV) variant that efficiently infects adult neural stem cells in the subventricular zone. The novel AAV, SCH9, was selected from a shuffled library of over 1.6 million variants that was designed using the computational algorithm SCHEMA. SCH9 mediates 24-fold higher EGFP expression and 12-fold greater transduction volume than AAV9 in the SVZ, and displays improved specificity for adult neural stem cells. Interestingly, we found that SCH9 combines properties from multiple AAV parent serotypes in its ability to utilize both galactose and heparan sulfate as receptors for cell transduction. Moreover, SCH9 is less susceptible to neutralizing antibodies than the AAV serotypes from which it is derived. Taken together, these results establish SCH9 as an effective gene delivery vector to study and manipulate the subventricular neurogenic niche.

507. An Alternative Route of Infection for a Novel AAV

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Viral vector mediated gene delivery has been proposed as a therapy for cystic fibrosis lung disease. Adeno-Associated Virus (AAV) vectors offer great potential as they are tractable and minimally immunogenic. Evidence from clinical trials suggests AAV2 vectors are safe, but transduce primary human airway epithelia (HAE) inefficiently. This drawback is thought to be due to a lack of appropriate binding partners on the apical membrane. Previous efforts derived a novel vector from AAV serotypes 2 and 5 using a directed evolution strategy. The novel vector AAV2/2.5T outperformed parental serotypes by orders of magnitude when applied apically to HAE. Further, binding assays demonstrated that unlike AAV2 the binding of AAV2/2.5T is specific, and when compared to AAV5 a greater number of AAV2/2.5T capsids need to be applied to saturate available binding sites. Recently, the transmembrane protein KIAA0319L was shown to be the essential receptor for all naturally occurring AAV serotypes and renamed AAVR, but it is uncharacterized in airway cells. We hypothesized AAVR was not expressed on the apical membrane of HAE, and that AAV2/2.5T was evolved to apically bind and infect HAE using a receptor other than AAVR. The goals of this study were to 1) establish whether HAE express AAVR, 2) discover the localization of AAVR in HAE, and 3) determine if blocking AAVR alters AAV2/2.5T infection. RNA sequencing and Western bolt analysis indicated that AAVR was expressed in HAE cells. Immunofluorescence of AAVR did not show apical membrane localization, but rather a predominant localization to a basolateral microdomain. Measured with a green fluorescent protein (GFP) transgene reporter, anti-AAVR antibodies did not reduce AAV2/2.5T transduction of HAE on the apical membrane. Antibodies slightly reduced basolateral transduction of HAE by AAV2/2.5T, but virtually eliminated basolateral transduction by AAV2. In HEK293T cells, AAV2 produced a more efficient infection than AAV2/2.5T and AAV5, in HeLa cells however AAV2/2.5T outperformed parental strains. In both cell lines, anti-AAVR antibodies blocked infection by all vectors in a dose dependent manner, with the highest doses reducing GFP fluorescence to near background levels for AAV2 and AAV5. High dose antibodies similarly blocked AAV2/2.5T infection on HEK293T cells, but could not decrease infection to near background levels on HeLa cells. These findings suggest that 1) AAV2/2.5T has expanded infectious capabilities on the apical surface of airway cells, 2) These capabilities are not due to the presence of AAVR at the apical membrane of HAE, 3) AAV2/2.5T retains the ability to utilize AAVR when applied to the basolateral membrane of HAE, and when applied to non-polarized, immortalized cell lines such as HEK293T and HeLa, and 4) AAV2/2.5T also has enhanced infectious capabilities on HeLa cells, where infection is more efficient than parental serotypes and less susceptible to blocking with anti-AAVR antibodies. Taken together, these results suggest an alternative route of infection for AAV2/2.5T in HAE involving utilization of a receptor other than AAVR, and that this receptor may be highly expressed in HeLa cells.

508. A Barcode-Based In Vivo Screening Method for Creating Novel AAV Serotypes for CNS-Directed Gene Therapy

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Capsid modification is a useful strategy to create adeno-associated virus (AAV) vectors with subtype specific neuronal targeting and enhanced retrograde transport. Incorporating known cell-specific binding ligands is a rational method, but the creation of vectors without prior knowledge has the potential to reveal novel targets. Directed evolution

and phage display are broadly utilized high-throughput methods, but are inefficient due to displaying random peptides wherein the vast majority will be non-functional and require multiple generations of selection.

Here, we have developed a novel AAV library in which each virus particle display a peptide derived from known proteins on the surface of an AAV2 capsid. The packaged viral genome encodes a unique barcode sequence to facilitate capsid identification. 92398 unique oligos encoding 14-amino-acid peptides derived from 135 neuron-related proteins were synthesized using microarray. Four-fragment Gibson assembly and novel emulsion PCR was used to generate a plasmid library by inserting oligos into the capsid gene and barcodes between the inverted terminal repeats. This plasmid library was then used to assemble a diverse library of AAV capsids, such that particles were composed of only peptide-modified capsid proteins. The modified capsids packaged an expression cassette containing RNA expressed barcodes providing post hoc identification of the capsid structure. In parallel, the plasmid library was sequenced using Illumina paired-end sequencing to link the RNA expressed barcodes to the de novo capsid structures. The AAV library efficiently infected neurons and astrocytes *in vitro* and displayed efficient retrograde transport ability in neurons *in vivo* (e.g., transported from striatum to substantia nigra, amygdala and thalamus). Functional peptides, which successfully promoted neuronal infectivity or retrograde transport, were identified through linkage between Illumina sequenced plasmid library and RNA expressed barcodes from both *in vitro* and *in vivo* samples.

Validations of 25 novel capsids and 4 standard capsids revealed a number of new serotypes with unique features. Some showed increased infectivity in primary neurons, glial cells and human ES cells *in vitro* (e.g. MNM010, MNM017 & MNM026). Another serotype (MNM004) was validated *in vivo* by retrogradely activating neurons in the basolateral amygdala using chemogenetic DREADD's, which lead to increased anxiety in the animals. Furthermore, using a capsid selected from substantia nigra samples in the screening (MNM008), we could specifically target and infect dopaminergic neurons retrogradely from the striatum to the substantia nigra in a Th-Cre rat model.

In conclusion, we developed a high-throughput combinatorial method to generate peptide-modified AAV libraries that are valuable for evaluation of receptor expression of neuronal populations and have the potential to generate novel vectors with unique properties for *in vivo* gene transfer in the CNS.

In Vivo Gene Editing

509. *In Vivo* Genome Editing via Non-Viral Delivery of Zinc Finger Nucleases Results in Supraphysiological Levels of Therapeutic Proteins in Adult Mice

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Background: Lysosomal storage disorders and hemophilia are examples of monogenic disorders that may be treated by enzyme replacement therapies (ERT). However, ERT often requires frequent infusions and patients experience peaks and troughs of efficacy throughout the course of treatment due to the short plasma half-lives of these proteins. In contrast, ZFN-mediated genome editing potentially enables permanent hepatic expression of a therapeutic enzyme via a one-time treatment. We have previously presented on ZFN-mediated *in vivo* genome editing to phenotypically correct the lysosomal storage diseases MPS I and MPS II as well as hemophilia B in murine models where adeno-associated viral vectors (AAVs) were used for delivery of ZFNs and transgene donors. ZFNs targeted to intron 1 of the murine *Albumin* gene were co-delivered intravenously with a promoterless therapeutic transgene donor, *e.g.* a corrective copy of the *hIDUA*, *hIDS*, or *hFIX* gene (MPS I, MPS II, or hemophilia B, respectively). This treatment resulted in targeted hepatic insertion of the transgene into the highly expressed *Albumin* locus and led to secretion of supraphysiological levels of functional protein into peripheral circulation and uptake into secondary tissues. Method: In this study, we employ lipid nanoparticles (LNP), a novel delivery vehicle that enables repeat administration and is not limited by the presence of pre-existing neutralizing antibodies, to mediate delivery of ZFN mRNA into hepatocytes to enable *in vivo* genome editing. mRNA encoding ZFNs targeting the murine *Albumin* locus was co-delivered with an AAV vector comprising a promoterless therapeutic transgene donor (*hIDS* or *hFIX*) via the tail-vein route in wild type C57/Bl6 mice. Genome editing efficiency was assessed in liver tissue by deep sequencing, and plasma human iduronate 2 sulfate (hIDS) or human factor IX (hFIX) protein levels were assessed using a functional activity assay or ELISA assay, respectively. Results: We demonstrate that intravenous administration of albumin-specific ZFN-encoding mRNAs formulated into LNP results in high levels of genome editing (up to 47% indels) in the murine liver after a single dose (0.5 - 2 mg/kg of mRNA). Co-delivering the mRNA-LNP with AAV comprising either a promoterless human *IDS* or *FIX* transgene donor results in therapeutically-relevant levels of enzymatic activity (1950 nmol/hour/mL) and protein expression (1015 ng/mL), respectively, within the plasma (up to 7700-fold 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). Conclusions: These

results demonstrate LNP-mediated ZFN delivery can drive highly efficient levels of *in vivo* genome editing and can potentially offer a new treatment modality for monogenic diseases.

510. Non-Viral Delivery of Zinc Finger Nucleases Enable Greater Than 90% Protein Knockdown of Multiple Therapeutic Gene Targets *In Vivo*

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Zinc finger nucleases (ZFNs) are sequence-specific nucleases that 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 can induce high levels of genome editing (up to 47% indels) at the *Albumin* locus within the murine liver after a single intravenous dose (2 mg/kg). Importantly, repeat dosing was capable of yielding an accumulation of indels after each subsequent dose. Next, ZFNs were designed against the murine *TTR* and *PCSK9* coding regions, which are well-validated gene knockout/knockdown targets for treatment of transthyretin-related amyloidosis and hypercholesterolemia, respectively. Lead 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 LNPs, and injected into wildtype mice. After 2-3 doses (0.8 mg/kg), 61% and 32% indels in bulk liver tissue and 91% and 92% protein knockdown in plasma were observed for TTR and PCSK9, respectively. These levels of gene modification and protein knockdown were stable out to 77 days and all doses were well-tolerated. 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 monogenic diseases.

511. *In Vivo* ZFN-Mediated Editing of the Mutant *SERPINA1* Gene Results in Spontaneous Liver Repopulation by Gene-Edited Hepatocytes and Greatly Decreased Fibrosis in the PiZ Mouse Model of alpha-1 Antitrypsin Deficiency Liver Disease

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Background: α1-Antitrypsin (AAT) deficiency (ATD) is commonly caused by a mutation in exon 5 of *SERPINA1*, leading to the expression of an inefficiently secreted variant (ATZ). Reduced ATZ secretion causes emphysema owing to unmodulated neutrophil elastase activity in lung, whereas the hepatocellular stress due to ATZ accumulation causes liver disease. PiZ mice are transgenic for the mutant human *SERPINA1* (*SA1-ATZ*) and exhibit ATZ accumulation in hepatocytes and liver fibrosis. Wildtype (WT) mouse hepatocytes competitively repopulate the host liver after transplantion into PiZ mice. We hypothesized that disrupting ATZ expression and expressing WT AAT by genome editing *in vivo* would confer a proliferative advantage to the genome-edited hepatocytes, enabling them to repopulate the liver. **Method**: Two recombinant adeno-associated viruses (AAV) were generated to each express an individual zinc-finger nuclease (ZFN) from a pair targeting exon 5 of *SA1-ATZ* (AAV-ZFNs). Another AAV was designed to provide a WT donor DNA template (AAV-Donor) for correcting the SA1-ATZ gene by targeted insertion of the donor. PiZ mice were injected i.v. with AAV-Donor alone, or with AAV-ZFNs at a low dose ($7.5x10^{10}$, LD) or a high dose ($1.5x10^{11}$, HD), with or without AAV-Donor. Groups of mice were sacrificed 2 weeks and 6 months after treatment for molecular, histological, and biochemical analyses. **Results**: Two weeks after treatment, deep sequencing of the hepatic *SA1-ATZ* gene pool show 8±4% or 23±8% indels by non-homologous end joining (NHEJ) respectively in mice receiving LD or HD AAV-ZFNs. When the AAV-Donor was co-administered with LD or HD AAV-ZFNs, targeted insertion occurred at 0.25±0.2% and 0.5±0.4 of *SA1-ATZ* genes. Serum human ATZ levels declined by 30±6 and 40±5% in the LD and HD groups, respectively, compared with controls. Six months after treatment, the percentage of *SA1-ATZ* genes with indels increased to 64±8% and 58±20% of recipients of LD or HD AAV-ZFNs, respectively. In mice receiving HD AAV-ZFNs plus AAV-Donor, up to 1.7% of *SA1-ATZ* genes were corrected by targeted insertion of the donor. In parallel, serum ATZ levels declined by 47% and 70% in the LD and HD AAV-ZFNs recipients, respectively, and liver fibrosis, was greatly reduced compared with controls. **Conclusion:** ZFN-mediated editing of the *SA1-ATZ* transgene *in vivo* and insertion of the corrective

donor provides a proliferative advantage to PiZ mouse hepatocytes, allowing them to preferentially repopulate the liver and reverse hepatic fibrosis, suggesting its therapeutic potential for treatment of ATD.

512. *In Vivo* Selection of Engineered Human CD34$^+$ HSPCs Using Targeted Gene Integration

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Targeted genome engineering is a rapidly growing field in precision medicine in which diseases are treated at the genetic level. The ability to manipulate a specific target DNA sequence includes knocking out expression of an unwanted gene, correcting a malfunctioning gene, or even inserting an exogenous gene. Historically, when new genes have been introduced into a cell population of interest, engineered retroviral vectors were reagent of choice. These vectors are able to permanently integrate genes into the cells chromosomal DNA and have proved invaluable in the treatment of many diseases. However, due to the random nature of integration site selection, the potential of integrations occurring near proto-oncogenes is an ongoing concern.

A more recent approach to the field of gene therapy is the use of nuclease-mediated DNA breaks to elicit the homology directed repair (HDR) pathway, where a false chromatid donor template can be used to insert specific sequences into a desired chromosomal location. Several such ideal sites have been identified as potential 'safe-harbor' loci, where the targeted integration (TI) and expression of new genes has not been observed to perturb local gene expression or lead to transformation.

The challenge with TI in human CD34$^+$ hematopoietic stem and progenitor cells (HSPCs) has been achieving persistent levels of modified cells high enough to observe a therapeutic benefit. To ensure we are engineering true, long-term HSPCs, we have utilized a new panel of surface markers to identify these cells and to monitor for TI events in the most primitive fraction. Additionally, in order to circumvent potentially low levels of targeted integration into CD34$^+$ HSPCs, we have also included a selectable marker into the integration cassette to enable *in vivo* selection of modified cells.

In order to test the engraftment potential and hematopoietic development of engineered CD34$^+$ HSPCs, we used the humanized NSG mouse model. By combining specifically designed nucleases (ZFNs) with AAV-delivered donor template DNA, we have achieved upwards of 30% of cells containing an integrated 2kb fragment expressing both GFP and our selectable marker. These HSPCs were infused into NSG mice and led to multi-lineage engraftment of the engineered cells. Mice then received a low dose of the selectable drug to enable *in vivo* selection of modified cells, resulting in a 2-3x fold increase of these modified cells in the peripheral blood and over 12% of engrafted CD34$^+$ cells in bone marrow containing the integration cassette. Additionally, using next-generation sequencing, we are able to track unique clones over time in peripheral blood and tissues.

In these studies, we have for the first time specifically engineered HSPCs with a large integration cassette, and enabled direct *in vivo* selection of modified cells after transplant. These cells engraft normally and undergo multi-lineage hematopoietic development in mice, can be clonally tracked, and subsequently selected for *in vivo*. This novel approach enables a straightforward method for increasing the number of edited cells in precision medicine to achieve a desired or therapeutic level of gene modification.

513. CRISPR/Cas9-Mediated *In Vivo* Gene Targeting Corrects OTC Deficiency in the Newborn OTC spf^{ash} Mice

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Many genetic metabolic diseases, including ornithine transcarbamylase (OTC) deficiency, present in newborns with repeated metabolic crises that can be lethal. Adeno-associated virus (AAV) neonatal gene therapy would require multiple vector administrations to maintain therapeutic effects, because the non-integrating genome is lost as hepatocytes proliferate. We recently developed a dual-AAV vector approach for *in vivo* delivery of three key components for CRISPR/Cas9-mediated homology directed repair (HDR): Cas9 enzyme from *Staphylococcus aureus* (SaCas9), a single-guide RNA (sgRNA), and a donor template. We demonstrated HDR-based correction of the G-to-A mutation in 10% of OTC alleles in the liver of newborn spf-ash mice and clinical benefits following *in vivo* genome editing. Since most monogenic genetic diseases are caused by different mutations scattered throughout a specific gene, a vector developed for one mutation would not be applicable for a patient with a different mutation. In this study, we aimed to develop a more universal CRISPR/Cas9 gene targeting approach in which the vector system could be applied to almost all patients with a specific disease. We developed a two-vector approach to incorporate all three components of CRISPR/Cas9 into AAV for targeted insertion of a human OTC transgene cassette into intron 4 of the mouse OTC locus. Vector 1 expresses the SaCas9 gene from a liver-specific TBG promoter. In addition to the sgRNA sequence expressed from the U6 promoter, vector 2 contains a TBG.hOTCco.bGH cassette flanked by 0.9 kb donor fragments on each side. Untargeted control vector 2 contains all components except for the 20 nt target sequence. Spf-ash pups were injected intravenously on postnatal day 2 with mixtures of vector 1 and vector 2, and subsequently evaluated for OTC expression and functional correction of OTC deficiency. At 3 and 8 weeks post vector co-administration, analyses showed 26% OTC functional-positive cells in liver, which was 3-fold higher than untargeted controls. At 8 weeks, the rate for HDR-mediated targeted insertion of the hOTCco cassette was an average of 27% compared to 0.3% in untargeted controls. We further assessed the impact of

gene targeting on the clinical manifestations of OTC deficiency by evaluating the tolerance of spf-ash mice to a one-week course of high-protein diet. Spf-ash mice treated with the gene targeting vectors had significantly lower plasma ammonia levels and showed a survival improvement compared to untreated or untargeted spf-ash mice. This study provides convincing evidence for efficacy in an authentic animal model of a lethal human metabolic disease following *in vivo* CRISPR/Cas9-mediated gene targeting.

514. Self-Destructing CRISPR-Constructs for Targeted Genome Editing in the Retina

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Delivery of CRISPR/Cas proteins remains one of the major remaining impediments to the widespread application of *in vivo* gene editing and the anticipatory cures of monogenic retinal diseases. We recently reported the utility of viral mediated CRISPR/Cas gene editing in the retina; however, with such a viral delivery system, active exogenous endonucleases will be maintained in the retina for an extended period. Consequently, CRISPR/Cas genotoxicity is a significant consideration in clinical applications. To address this issue, we have rationally designed a self-destructing "kamikaze" CRISPR/Cas system that disrupts the CRISPR/Cas enzyme itself after active protein expressed. Four different guide RNAs (sgRNAs) were designed to target Streptococcus pyogenes Cas9 (SpCas) and after *in vivo* validation, selected sgRNAs were cloned into the pX552-YFP-sgRNA plasmid to generate yellow fluorescent protein (YFP) targeting "kamikaze-CRISPR/Cas" vector (pX552-YFP sgRNA-SpCas9 sgRNA). The editing efficacy our "YFP targeting kamikaze-CRISPR/Cas" constructs were further validated *in vitro* in YFP expressed HEK293 cells by Western blot and FACS analysis. Constructs were packaged into an adeno-associated virus 2 (AAV2) vector and administered intravitreally in the *Thy1-YFP* transgenic mouse and the expression of SpCas9 as well as number of YFP fluorescent cells in the retinas were evaluated at 8 weeks after injection. AAV2-mediated single YFP or LacZ targeting SpCas9 sgRNAs were used as controls.Marked reduction of SpCas9 protein concentration (90% at day 2) as well as YFP expression (80% at day 10) was found following transfection of our YFP targeting kamikaze-CRISPR/Cas vector in YFP-expressing HEK293 cells compared to the LacZ targeting CRISPR/Cas control. AAV2-mediated *in vivo* delivery of this construct significantly reduced the number of YFP fluorescent cells (86% reduction) in the inner retina of *Thy1-YFP* transgenic mice. Moreover, a significant decrease of SpCas9 mRNA was also detected in AAV2-mediated YFP targeting kamikaze-CRISPR/Cas infected retina over relative to those treated with YFP targeting CRISPR/Cas alone. CRISPR/Cas genotoxicity is a significant consideration in future clinical applications. Our work has demonstrated that a "self-destructing/kamikaze" CRISPR/Cas can be used as a safe and robust tool for refined gene editing in the retina.

515. Nanoparticle Delivery of Cas9 Ribonucleoprotein and Donor DNA *In Vivo*

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Abstract Cas9 based therapeutics have the potential to revolutionize the treatment of genetic diseases because of their ability to generate homologous DNA recombination (HDR) and correct DNA mutations. However, viral gene therapy is currently the only delivery technology available for generating HDR *in vivo* with Cas9, and is challenging to bring into clinical trials because of off-target DNA damage and immunogenicity. In this report, we present a non-viral Cas9 delivery vehicle, termed CRISPR-Gold, which can for the first time induce HDR *in vivo* by directly delivering Cas9 protein, gRNA, and donor DNA. CRISPR-Gold is composed of gold nanoparticles assembled with the Cas9/gRNA ribonucleoprotein (RNP) complex, donor DNA, and an endosomal disruptive polymer. We show here that CRISPR-Gold can correct the DNA mutation that causes Duchenne muscular dystrophy (DMD) in mdx mice via HDR, with an efficiency of 5.4% after an intramuscular injection and with minimal levels off-target DNA damage. In addition, we demonstrate that CRISPR-Gold can improve muscle strength and lower tissue fibrosis in mdx mice. CRISPR-Gold is the first example of a DMD therapeutic that can correct the mutated dystrophin protein back to its wild type sequence, and has tremendous potential for treating DMD and other genetic diseases caused by single base pair mutations.

516. CRISPR-Mediated Integration of Large Gene Cassettes Using AAV Donor Vectors

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Precise genome editing can be accomplished using designer nucleases (e.g. ZFNs and TALENs) or the CRISPR/Cas9 system, which create site-specific double-strand DNA breaks that stimulate homologous recombination (HR) when supplied with a homologous donor DNA template. This method can facilitate targeted integration of transgenes for gene therapy or studies of gene function.

Viral vectors derived from the non-pathogenic, single-stranded DNA virus, Adeno-Associated Virus (AAV), can transduce both dividing and non-dividing cells. Recently, we and others have shown that AAV vectors can very efficiently serve as template DNA during precise genome editing by HR in human primary cells, including T cells, CD34+ hematopoietic stem and progenitor cells (HSPCs), and hepatocytes.

However, the maximum packaging capacity of AAV of approximately 4.5 kilobases limits applications of genome editing with AAV donor vectors since the homology arms required for efficient HR add a minimum of 2 x 0.4 kb to the vector, leaving 3.7 kb for promoter, transgene, and polyadenylation signal. Several genetic diseases involve mutations in genes that would be challenging to fit within this size and

the use of multi-cistronic cassettes are even further limited. Here we present a CRISPR/Cas9-based methodology that enables site-specific integration of large transgenes that are split between two AAV donor vectors.

Since HR is a seamless process, we envisaged that two parts of a large transgene could be fused together by consecutive HR events using two different AAV donor vectors. Using this approach, we demonstrate integration frequencies of both vectors in K562 cells, primary human T cells, and CD34+ HSPCs of up to 44%, 25%, and 12% in the three cell types, respectively. Importantly, genotyping of HSPC-derived methylcellulose clones confirm precise and seamless integration of both donors. Furthermore, we show that CD34+ HSPCs edited using this approach have long-term (>16 weeks) repopulation capacity in immunodeficient mice. Key aspects of the system is that it uses only a single sgRNA and it does not involve having to serially transfect and transduce cells, but instead can be performed in one step, during which the intracellular homologous recombination machinery naturally iterates the process.

In conclusion, we believe this methodology is widely applicable for site-specific integration of large transgenes or multicistronic expression cassettes in primary human cells, which will enable a range of therapeutic and research applications.

Immunological Aspects of Gene Therapy and Vaccines

517. Predictors of Response to CD19-Specific CAR T Cell Therapy

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Tolerance to self-antigens is a major barrier to the elimination of human cancer by the immune system. We redirected T cells from patients with CLL against leukemic cells by engineering with a CD19-specific chimeric antigen receptor (CAR) signaling through 4-1BB and CD3ζ (CTL019). We previously demonstrated that such CTL019 cells can induce long-term remissions in CLL, but in only a subset of subjects (PMID: 26333935). To date, little is known about predictive indicators of CAR T cell efficacy. This study was designed to evaluate biomarkers of clinical response to CTL019 in CLL. Here we show that *in vivo* expansion and persistence are key quality attributes of infused CTL019 cells in CLL patients (n = 42) who have complete responses (CR) to therapy, which are ongoing past six years in two cases and accompanied by the presence of functional CTL019 cells. Furthermore, durable remissions are associated with transcriptomic signatures of early memory T cells in the infusion product, while T cells from non-responding (NR) patients are enriched in genes belonging to known pathways of terminal differentiation and exhaustion. These findings were confirmed via polychromatic flow cytometry and showed a dramatically increased proportion of T cells expressing PD1 alone and the combination of PD1 with TIM3 or PD1 and LAG3 in NR patients. Flow cytometric analysis also revealed enrichment of not the canonical naïve/memory T cell subsets, but of CD45RO-CD27+ CD8+ T cells at the time of apheresis. Re-stimulation of the cellular infusion product through the CAR further demonstrated that CTL019 cells from CR patients produced significantly higher levels of several cytokines compared with NR patients, including IL-21, IL-22, IL-17, and IL-6, suggesting that the STAT3 signaling pathway may be involved in the maintenance of T cell memory and potentiation of anti-tumor efficacy. These findings underscore the potential utility of increasing the therapeutic efficacy of adoptively-transferred CAR T cells by selecting cultures with high absolute numbers of less differentiated and less exhausted lymphocytes possessing enhanced proliferative and survival capacity. The biomarkers here identified in pre-manufacturing T cells may serve to further target likely responder patients; for that purpose we have now extensively validated a biomarker assay which we plan to roll out in a future CAR T cell trial.

518. Antigen-Specific Regulatory T Cells Generated by Factor VIII-CAR Retroviruses Transduction Suppress Anti-Factor VIII Immune Responses

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The formation of inhibitory antibodies to factor VIII (FVIII; F8 in constructs) protein is a major challenge for effective treatment for hemophilia A (HemA) patients. In our previous studies, the expanded FVIII-specific regulatory T cells (Tregs) from polyclonal $CD4^+$ splenocyte exerted better suppressive function compared with non-specific Tregs. However, the number of FVIII-specific Treg is small in polyclonal $CD4^+$ T cells population. In order to obtain the stable source of antigen-specific T cells, we adopted chimeric antigen receptor (CAR) approach to generate antigen-specific Tregs. The CAR construct included high-binding anti-FVIII antibody-derived variable region (scFV) linked to signaling and costimulatory moieties of immune receptors and 2A peptide fused with a murine Foxp3 cDNA (F8CAR-mFoxp3). In our earlier results, the F8CAR-mFoxp3-lentivirus (LV) transduced murine T cells inhibited the F8-specific effector T cells (Teff) proliferation in an *in vitro* suppressive assay. Nevertheless, the transduction efficiency reached only 16% due to inefficient entry of

lentivirus into murine CD4+ T cells. We tested a variety of transduction methods carrying the CAR construct in different vectors. The Eco-retrovirus (RV) produced the best F8CAR transduction efficiency up to 60% in murine CD4+ T cells. The transduced T cells were further selected by antibiotics, expanded using anti-CD3/anti-CD28beads, and stained positive for F8CAR and Foxp3 expression by flow cytometry. *In vitro* suppressive assay by co-culturing F8CAR-mFoxp3-RV engineered CD4+ T cells and FVIII-specific Teffs from FVIII-primed HemA mice indicated that the retrovirus engineered Tregs suppressed the proliferation of FVIII-specific Teffs. In addition, we generated the FVIII-specific Teffs by transducing CD4+ T cells with F8CAR-RV and selected with antibiotics. With purified F8CAR Teffs, a CFSE assay can be performed and showed that F8CAR-mFoxp3 Tregs significantly suppressed the proliferation of F8CAR Teffs in an antigen-specific fashion. Subsequently, we performed the adoptive transfer experiment to examine the suppressive function of the engineered Tregs *in vivo*. $1x10^6$ F8CAR-mFoxp3-RV transduced cells and untransduced cells were injected into HemA mice. In addition, polyclonal Tregs isolated from FVIII-primed HemA mice and expanded antigen-specifically are injected to a separate group of control mice. One day after cell transfer, the treated mouse groups are challenged with FVIII plasmid injected hydrodynamically. The anti-FVIII antibody titers are evaluated overtime. F8CAR-Foxp3-RV transduced cells more significantly decrease the production of anti-FVIII antibodies in recipient HemA mice compared to the mice receiving untransduced T cells and polyclonally expanded FVIII-specific Tregs. The mice receiving RV engineered cells and control naive HemA mice are then challenged with a non-specific antigen and both groups of mice showed the same responses. Compared with nonspecific and polyclonally expanded Tregs, F8CAR-mFoxp3 Tregs exert superior suppressive activity towards anti-FVIII immune responses without triggering systemic immune suppression. These results demonstrate the potential of F8CAR-mFoxp3 engineered cells to modulate anti-FVIII immune responses.

519. A Novel Platform for Immune Tolerance Induction in Hemophilia A Mice

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Background: Hemophilia A (HA) is an X-linked bleeding disease due to factor VIII (FVIII) deficiency. Here we sought to restrict the expression of FVIII transgene in liver sinusoid endothelial cells (LSEC), the normal site of FVIII synthesis to reach long term expression and limit immune responses in mouse model by lentiviral (LV)-mediated gene therapy encoding FVIII forms under the control of cell-specific promoters. **Aims:** To study the role of LSEC in tolerance induction after HA gene transfer using lentiviral vectors (LV)-expressing FVIII driven by an endothelial-specific promoter ± miRNA target sequences (miRTs). **Methods:** We prepared LV containing BDD-hFVIII, RH-hFVIII or codon optimized hFVIII (co-hFVIII) cDNAs under the control of VEC (VE-cadherin; an endothelial marker) promoter alone or in combination with miRT142.3p (silencing in hemopoietic cells) and miRT122 (silencing in hepatocytes), and injected several HA mouse strains (C57Bl/6, B6/129 and BALB/c) naïve or previously immunized with FVIII. To study the role of LSEC in Tregs induction we depleted Tregs by anti-CD25 antibody injection before and after gene transfer *in vivo*. **Results:** C57Bl/6 HA mice injected with LV.VEC.FVIII±122-142 reached long-term phenotypic correction up to 1y with FVIII activity up to 12%, when more active forms of hFVIII were used. When we injected LV.VEC.FVIII±122-142 in FVIII-immunized mice we observed FVIII activity and reversion of inhibitor titers. Tregs depletion before gene transfer resulted in very low levels of FVIII and inhibitors, while depleting Tregs after gene transfer we observed an initial decline in FVIII activity and formation of inhibitors followed by recover of FVIII activity and reduction of inhibitor titers. The reversion of inhibitor titers in FVIII-immunized or Tregs-depleted mice was obtained even in absence of miRTs combination. In summary, we first report the expression of FVIII expression on its natural site of production with therapeutic levels of the transgene without formation of neutralizing antibodies (inhibitors) to FVIII. Moreover in previously FVIII-immunized HA mice with FVIII inhibitors were eradicated in a regulatory T cell-dependent mechanism. Finally, injection of LV.VEC.FVIII in two different HA immunereactive mouse strains, B6/129 and BALB/c, resulted in long-term FVIII activity and absence of anti-FVIII antibodies. **Conclusion:** In conclusion, LVs expressing FVIII under cell-specific promoters to target LSEC were able to overcome FVIII off-target expression, limiting immune responses and providing long-term phenotypic correction in HA mice. Thus, for some transgene, expression on the natural site of synthesis provides both efficacy and safety for long term correction of hemophilia and likely to others genetic disease.

520. Type I Interferon Signaling Is Essential for Cross-Presentation and Cross-Priming of CD8+ T Cells Against AAV Capsid

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AAV-mediated gene therapy continues to show tremendous promise to treat monogenic disorders such as hemophilia, but CD8+ T cells specific for the AAV capsid have impeded clinic progress. Our data suggest that activation of anti-capsid CD8+ T cells begins with TLR9-sensing of the vector's DNA genome, thus providing an activation signal to dendritic cells (DCs). The now activated DCs present AAV capsid antigen, leading to effective cross-priming of CD8+ T cells. Our previous work showed that two subsets of DCs, plasmacytoid DCs (pDCs) and conventional DCs (cDCs), were both required to prime anti-capsid CD8+ T cells but with discrete roles. Innate sensing through TLR9 was dispensable in cDCs for cross-priming but was required in pDCs while cDCs were required to cross-present capsid antigen to naïve CD8+ T cells. These findings prompted a model where innate sensing through TLR9 occurred in pDCs which *trans*-activated cDCs to get efficient cross-presentation and T cell priming. Type I interferons (T1 IFNs) are abundantly produced by pDCs following TLR9 signaling and are capable of promoting phenotypic maturation

and cross-presentation in cDCs. Here, we investigate the relationship between cDCs and pDCs in cross-priming anti-capsid CD8+ T cells in response to AAV, and test the hypothesis that T1 IFNs produced by pDCs following TLR9 ligation are an essential component to promote cDC licensing to cross-prime anti-capsid CD8+ T cells. In order to measure capsid-specific T cell responses, we have created a modified AAV2 capsid that contains the peptide sequence SIINFEKL (AAV2-SIINFEKL), which is the CD8+ immunodominant epitope of the model antigen ovalbumin in C57BL/6 mice. With this vector, cross-priming of capsid-specific CD8+ T cells can be measured using H2-Kb-SIINFEKL tetramer, and cross-presentation can be measured by proliferation of adoptively transferred T cells from TCR transgenic OT-1 mice. We first determined whether pDCs were required for cross-presentation and cross-priming using a BDCA2-DTR mouse model that allows for the selective depletion of pDCs. Mice were injected i.m. with AAV2-SIINFEKL ($1x10^{11}$ vg) and at both 7 and 10 days later, anti-capsid CD8+ T cell priming was abrogated in the absence of pDCs (n=4/group). However, proliferation of OT-I cells adoptively transferred 2 days post AAV2-SIINFEKL injection was only mildly reduced suggesting that pDCs are less required for cross-presentation. Next, to determine the effect of T1 IFNs on cross-presentation and cross-priming, we injected WT C57BL/6 mice (n=4/group) with PBS or a monoclonal antibody that blocks the T1 IFN receptor (α-IFNAR-1) 1 day prior to i.m. injection of AAV2-SIINFEKL. After 7 days, capsid specific CD8+ T cells were nearly absent in mice treated with α-IFNAR-1, while PBS injected controls showed the expected level of tetramer+ CD8+ T cells. In addition, proliferation of OT-I cells adoptively transferred 2 days p.i. into mice treated with α-IFNAR-1 was ~7% compared to a significantly higher ~17% in WT mice (n=4/group). Importantly, blocking the T1 IFN receptor did not inherently affect the ability of the OT-I T cells to proliferate, which readily occurred in response to peptide antigen. Together these results indicate that T1 IFN signaling is required for both cross-presentation and cross-priming of anti-capsid CD8+ T cells, while pDCs seem more specifically required for cross-priming and may not be the only source for T1 IFN.

521. Modulation of AAV Vector Dosing and Avoidance of Capsid Immune Responses via Repeated Co-Administration of Vector with Rapamycin Tolerogenic Nanoparticles

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Gene transfer approaches based on the adeno-associated virus (AAV) vector platform have shown great therapeutic potential in both preclinical studies and clinical trials. Neutralizing immune responses to AAV, however, are an important limitation to the use of AAV vectors, as anti-capsid neutralizing antibodies (NAb) develop at high titers following vector administration and persist for several years after AAV vector administration, making vector readministration not effective. We tested a novel strategy to modulate immune responses directed against AAV vectors based on the co-administration of biodegradable tolerogenic poly(lactic acid co-glycolytic acid) nanoparticles containing rapamycin (SVP) at the time of vector administration. We showed that co-administration of vector with SVP completely blocked anti-AAV immune responses in mice and allowed for vector readministration. Modulation of AAV vector immunogenicity appeared to be antigen-specific and depletion experiments suggest a role of regulatory T cells as mediators of this effect. Notably, treated animals did not appear to be immunosuppressed as they responded to immune challenges with alternate antigens. Additionally, SVP co-administration also effectively controlled memory responses to AAV and capsid CD8+ T cell responses triggered in the context of AAV gene transfer, both against capsid and transgene. Based on these promising results, scale up to non-human primates was performed. Animals received two administrations of AAV8 vectors carrying two different transgenes at 2E12 vg/kg, given one month apart. Vectors were administered intravenously with SVP or with empty nanoparticles as control. Results obtained indicate that SVP allow for safe and efficient vector readministration in a large animal model of gene transfer. Additional studies in hemophilia B mice, in which animals were dosed with either 2E11 vg/kg or an AAV8 encoding for coagulation factor IX (AAV8-FIX) or with the same vector dose split into two administrations, resulted in identical correction of the disease phenotype, demonstrating that SVP allow for modulation of vector dosing via repeated vector administrations. Similarly, using a secreted reporter transgene, up to 3 sequential dosing of vector were given, resulting in significant increase in transgene expression and control of capsid humoral immune responses. In conclusion, SVP administration at the time of in vivo gene transfer modulates AAV vector immunogenicity and allow for safe and effective redosing, thus addressing some of the most important challenges of gene transfer with this therapeutic platform.

522. Review of CSF and Peripheral Immune Responses Following Intrathecal Gene Transfer for Giant Axonal Neuropathy Intrathecal Gene Transfer for Giant Axonal Neuropathy

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OBJECTIVE: GAN is a rare pediatric autosomal recessive neurodegenerative disorder affecting the central and peripheral nervous system. Mutations in the *GAN* gene cause loss of function of gigaxonin, a cytoskeletal regulatory protein, clinically leading to progressive sensorimotor neuropathy, optic neuropathy, reduced coordination, and progressive respiratory failure with death by the 2nd to 3rd decade of life. We are currently conducting a first-in-human intrathecal (IT) AAV9 mediated gene transfer trial for GAN (NCT02362438). The immunologic consequence of IT gene transfer is not thus far known, but is relevant to other disorders where a similar approach to gene transfer may be sought. We present immunologic data, including neutralizing antibody (Nab) quantification in serum and CSF, and ELISPOT data for AAV9 and gigaxonin, and compare this to CSF cell counts and clinical assessments of safety. METHODS: Six patients with GAN (with at least one missense mutation in the GAN gene) have been dosed at all three dose levels (ranging from 3.5×10^{13} vg to 1.8×10^{14} vg) with scAAV9-JeT-GAN. Patients are pretreated with IV methylprednisolone, followed by a 4 week course of oral prednisone. Clinical safety is assessed by neurologic exam, neuroimaging, serum and CSF studies, and EKG/ 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) is performed at various time points. One additional 'null' mutation patient has been injected in the middle dose level with additional immunosuppression including with prednisone, tacrolimus, and sirolimus to minimize a potential higher risk for an adaptive anti-transgene response. RESULTS: 3 of 7 patients had positive preexisting serum NAb titers serum but none had positive NAb titers in CSF at baseline. There is a rise in both CSF and serum NAb levels following IT gene transfer. ELISpot analysis shows a T cell response to AAV9 but not to gigaxonin, that peaks by around 3 months and then declines spontaneously. There is a clinically asymptomatic mild concomitant inflammatory response seen on CSF examination, but without any corresponding change on exam, imaging, or serum chemistries. CONCLUSIONS: The phase I IT AAV9 mediated gene transfer study for GAN will establish proof of principle of this novel route of administration. Investigation of immune profiles following gene transfer may shed light on the safety of this route of gene transfer, dose limitations, and the role of elevated pre-existing NAbs with IT gene transfer. Further, while the IT gene transfer trial is focused primarily on patients with missense mutations, the trial is also investigating the use of a novel immunosuppressive protocol to address patients with null mutations, who should be considered "immune naïve".

523. *In vivo* Persistence of Gene-Modified, Antibody-Secreting Hematopoietic Cells

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There has been considerable progress in the treatment of HIV with antiretroviral therapy (ART), however, the replication-competent latent viral reservoirs persist in sanctuaries, leading to viral rebound after treatment interruption. Combinatorial treatment approaches will likely be necessary to limit the viral reservoir seeding during the acute phase of the infection and/or prevent virus reactivation following ART withdrawal. The recently discovered potent broadly neutralizing antibodies (bNAbs) represent a promising novel treatment strategy. bNAbs can neutralize the virus, stimulate the host immune responses such as antibody-dependent cell-mediated cytotoxicity and target both free virions and HIV infected cells. Several clinical trials are in progress demonstrating the safe administration and efficiency of these antibodies in passive transfer strategies but sustained expression still has to be achieved. Hematopoietic stem and progenitor cells (HSPCs) represent an appealing alternative to address these issues. Their indefinite renewal and differentiation into lineages migrating to tissues hosting the viral reservoirs, such as the central nervous system, could be key to sustained antibody secretion and delivery to the sanctuaries.

Using a lentiviral vector we have examined the ability to genetically modify human $CD34^+$ cells to support the secretion of bNAbs *ex vivo* and *in vivo*. We found that $CD34^+$ derived HSPCs were able to secrete PGT128, VRC01 and PG16 bNAbs antibodies up to 150 ng/ml *ex vivo*. We show that the colony-forming cell potential of the antibody-producing cells was similar to the unmodified cells. Following these observations, NOD-SCID-gamma (NSG) neonate mice were infused with lentivirally transduced human $CD34^+$ cells producing PGT128 or VRC01 bNAbs. Engraftments were similar between the mock and antibody-producing mice (Figure 1). The gene-modified cells engrafted efficiently *in vivo* and persisted in the peripheral blood for over eight months following the infusion. Gene-marked cells constituted CD3, CD4, CD8, CD14 and CD20 positive cells in the peripheral blood but were also found in the bone marrow, spleen and thymus at the time of necropsy, confirming the engraftment. The bNAbs transgenes were efficiently secreted in the serum starting around 14-16 weeks post-infusion until the end of the experiment. Antibody secretion was even detected in the serum of mice demonstrating a low percentage of gene-modified cells, indicating that a small number of antibody-producing cells is sufficient for antibody secretion. Protection against HIV challenge is now being analyzed.

The ability of the gene-modified antibody-producing hematopoietic cells to engraft, differentiate *in vivo* in the humanized mouse model and to sustainably secrete antibodies highlight the promising potential of using HSPCs as a means to deliver therapeutics. This strategy may also allow improved treatment approaches for lysosomal storage diseases by delivering and secreting a functional enzyme to the affected tissues. Future development will focus on optimizing the secretion and analyzing trafficking to the reservoir compartments. Importantly, this approach could be applied to other diseases in which current treatment include antibody expression, but require multiple injections for sustainable efficiency.

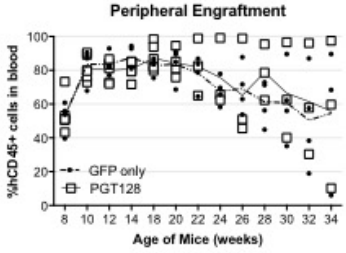

Figure 1: Engraftment as measured by the percentage of human CD45$^+$ cells detected by flow cytometry in the peripheral blood of mice humanized with mock (GFP only) or PGT128 antibody-producing cells at the indicated time points.

524. Zmapp Antibody Gene Transfer Mediated by Adeno-Associated Virus Vectors Protects Against Ebola Infections in a Murine Model

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The cocktail of Zmapp Ebola virus (EBOV) neutralizing antibodies is a very promising treatment for Ebola disease. Although Zmapp can quickly neutralize EBOV, it might not be very effective in prophylaxis because of the clearance of the antibodies. Recombinant vectors derived from the adeno-associated virus (rAAVs) could be useful to express Zmapp antibodies because they 1) can express antibodies at a therapeutic level for months; 2) are well tolerated and; 3) can transduce efficiently almost any tissue. In this study, we aimed to develop a prophylactic gene therapy for Ebola disease based on rAAVs to deliver the genes coding the Zmapp neutralizing antibodies (c2G4, c4G7 and c13C6). Furthermore, this treatment should also work in all individuals including those that are immunocompromised. The light and heavy chains of antibodies were expressed under the same expression cassette by using sequences for peptide 2A and an optimized furin cleavage site. Three rAAVs (serotype 9) expressing one of three antibodies under the control of a CAG promoter were produced in WAVE bioreactors up to 10L-scale. Production was accomplished by plasmid transfection using our patented cGMP compatible HEK293 cell line that grows in suspension and in serum-free medium. Produced rAAV9 were then purified by an iodixanol-step gradient and the genomes copies (VG) were quantified by qPCR. To determine the best route of administration, 10 mice per group were treated with rAAV9-c2G4 intravenously (IV), intramuscularly (IM) and intranasally (IN) at two different doses: $2.7x10^{10}$ and $1.3x10^{11}$ VG. Mice were then challenged with an intraperitoneal EBOV dose of 1000 LD_{50} twenty days later. 80% and 50% of the mice survived the challenge at the highest IV and IM dose, respectively. The treatment failed to protect the mice in the IN group, most likely due to an immune response against the chimeric antibody. Indeed, serum concentrations of anti-c2G4 antibodies were the highest in this group and higher in the serum of the treated mice from IV and IM groups that have failed the challenge. Furthermore, a concentration of at least 5 µg/mL of c2G4 was sufficient to protect mice against EBOV challenge. A second experiment was performed using a higher dose of $3.0x10^{11}$ VG. Interestingly, at this dose, 100% and 90% of the mice survived in the IV and IM groups, respectively. Separate IV injections of rAAV9-c4G7 and rAAV9-c13C6 successfully protected 70% and 30% of the mice, respectively. The cocktail of the three rAAV9 (rAAV9-Zmapp) was also tested at the same total dose ($3x10^{11}$ VG) i.e. one third of the dose for each rAAV9 (10^{11} VG per rAAV9). The protection efficacy reached 90% and 60% of the mice in the IV and IM groups, respectively. In conclusion, the injection of AAV-c2G4 alone and the rAAV9-Zmapp cocktail at the highest dose allowed an efficient protection despite the presence of an antibody-specific humoral response. Such promising prophylactic treatment for EBOV infection should be tested in larger animal models and different strategies should be evaluated to increase the antibody expression and to reduce the immune response against the Zmapp antibodies. The cocktail rAAV9-Zmapp could be useful to provide a sustainable protection for the first line workers or to protect susceptible individuals to control an outbreak following protocols based on ring vaccination.

Gene Therapies for Genetic and Metabolic Disorders

525. Manipulation of miR-143 Regulates Transfer of a Lysosomal Enzyme Across the Blood-Brain Barrier via Mannose 6-Phosphate Receptor

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Inefficient therapeutic delivery into the brain has been a major obstacle for the treatment of central nervous system (CNS) manifestations in lysosomal storage diseases (LSDs). The cation-independent mannose-6-phosphate receptor (M6PR) plays a critical role in lysosomal trafficking and intercellular transfer of most lysosomal enzymes, which is essential for metabolic cross correction in treating LSDs. Developmental decline of M6PR on blood-brain barrier (BBB) has been suggested in mice during early postnatal period; however, the underlying molecular mechanism remains to be elucidated. In this study, we documented a dramatic reduction of M6PR protein with slightly decreased M6PR mRNA in brain micro-vasculatures (BrMV) isolated from adult mice (>12 weeks of age) compared to those from pups (8-10 days of age). Microarray analysis of microRNAs (miRNAs) in BrMV identified 16 miRNAs with up-regulation and 7 with down-regulation in adult mice. Together with TargetScan and miRanda analysis, miR-143 is the only up-regulated microRNA that has presumed targets in 3' untranslated

region (UTR) of M6PR. Significantly higher expression of miR-143 in adult BrMV than in pups was further validated by RT-qPCR, which was reversely correlated with M6PR protein levels. Direct interaction between M6PR 3'UTR and miR-143 was demonstrated using a dual luciferase reporter assay with site-mutagenesis. Using two human vascular endothelia cell lines with different endogenous levels of miR-143, downregulation of miR-143 by overexpressing a sponge cluster or upregulation by expressing pre-miR-143 from CMV promoter proved to reversely modulate endogenous M6PR protein levels. Such receptor variations were also correlated with changes in M6PR-mediated endocytosis of α-L- Iduronidase (IDUA), a lysosomal enzyme associated with Mucopolysaccharidosis type I (MPS I), a neurological lysosomal storage disease. Moreover, the removal of miR-143 led to significant increase of M6PR expression in BrMV isolated from miR-143 knock-out mice (miR-143KO) compared to those from adult C57/Bl6 mice (WT) as determined by Western blot and immunofluorescent analysis. Enhanced cell-surface distribution of M6PR was evidenced in BrMV of miR-143KO, as compared to barely detectable levels in WT mice using *ex vivo* antibody binding and internalization assay. To evaluate therapeutic potential of downregulating miR-143 on M6PR-mediated CNS delivery, we generated a mouse model with miR-143 knock-out on a background of IDUA-deficient MPS I mice (MPS/miR-143KO). Two days after hydrodynamic injection of plasmid overexpressing IDUA from a hepatic-specific promoter, significantly higher IDUA activities were detected in capillary-depleted, enzyme-deficient brain parenchyma of MPS/miR-143KO mice than those of age-matched MPS I mice. Importantly, the accumulation of brain glycosaminoglycans and abnormally elevated β-hexosaminidase activities were normalized in treated MPS/miR-143KO, but not in MPS I mice, demonstrating therapeutic potentials with down-regulation of miR-143. Immunofluorescence analysis demonstrated wide-spread distribution of IDUA in neurons and activated microglia cells in cortex, middle brain and brainstem of MPS/miR-143KO but not of MPS I mice, indicating that loss of miR-143 facilitates IDUA delivery across the BBB. This study not only demonstrates functional regulation of M6PR-mediated protein transfer by miR-143 in vascular endothelial cells and in BBB of MPS/miR-143KO mice, but also provides the feasibility of a novel CNS-targeted approach that would be applicable in treating many neurologic LSDs involving M6PR pathway.

526. A Blood-Brain Barrier-Crossing Peptide and Lentiviral-Mediated Stem Cell Gene Therapy Correct Cognitive, Motor and Skeletal Impairment in a Mouse Model of Mucopolysaccharidosis Type II

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Mucopolysaccharidosis type II (MPS II) is an X-linked lysosomal storage disorder characterized by mutations in the iduronate-2-sulphatase (IDS) gene, which normally degrades complex sugars in lysosomes. These mutations lead to cellular accumulation of glycosaminoglycans in the brain and skeleton, and culminate in death by teenage years. Severe MPS II is non-responsive to enzyme replacement therapy or standard haematopoietic stem cell transplantation but remains the most frequent form of MPS II. Here, we report a novel lentiviral-mediated stem cell gene therapy approach to specifically target the brain using a blood-brain barrier (BBB)-targeting peptide. Haematopoietic stem cells (HSCs) were corrected using a lentiviral vector encoding the CD11b promoter and human IDS (LV.CD11b.IDS) or human IDS fused with a BBB peptide (LV.CD11b.IDS.Peptide), and transplanted into 6-8 week-old MPS II mice after full myelo-ablative conditioning using busulfan. LV.CD11b.IDS- and LV.CD11b.IDS.Peptide-transduced HSCs showed increases in IDS enzyme levels of 124-fold and 152-fold over wild-type, respectively, and complete engraftment into MPS II male mice at 8-weeks post-transplant. We achieved vector copy numbers in HSCs of 3.1 and 3.8 in LV.CD11b.IDS and LV.CD11b.IDS.Peptide groups, respectively. MPS II mice were evaluated for neurocognitive, skeletal and activity deficits using a series of behavioural tests and whole body x-rays at 8 months of age. We show complete correction of skeletal abnormalities and motor function using both lentiviral groups. Inflammatory cytokine profiles were normalised with LV.CD11b.IDS.Peptide in 8-months-old brains, and more importantly, cognitive deficits were fully corrected with LV.CD11b.IDS.Peptide but not LV.CD11b.IDS. These results provide excellent proof of principle for the translational outcomes of this approach for the treatment of the neurocognitive impairment in MPS II patients.

527. Overcoming Limitations Inherent in Sulfamidase to Improve MPS IIIA Gene Therapy

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A deficiency of the lysosomal hydrolase sulfamidase causes mucopolysaccharidosis type IIIA (MPS IIIA), a lysosomal storage disease that affects the brain. In earlier work in a different lysosomal storage disease, we exploited the utility of AAVs to transduce ventricular lining cells for secretion of enzyme into the cerebrospinal fluid (CSF), resulting in subsequent enzyme delivery throughout the brain and benefit on cognition and lifespan in mice and dog models. A critical feature of this approach is very efficient secretion of a significant proportion of the expressed enzyme from transduced cells. Because sulfamidase is not efficiently secreted, we performed site directed mutagenesis on the native enzyme, and identified variants that showed improved secretion over the wildtype enzyme following gene transfer. This variant also exhibited improved uptake properties that were mannose-6-P receptor independent. In studies in MPS IIIA deficient mice, we found that transduction of ventricular lining cells with AAVs expressing variant sulfamidase resulted in improved spatial learning, reduced memory deficits, and resolution of secondary lysosomal enzyme elevations in the CSF and brain parenchyma. These data contrasted those obtained using AAVs expressing wildtype sulfamidase, where secretion into the CSF was significantly lower and there were no significant improvements in behavior phenotypes. These results demonstrate the utility of a novel enzyme variant of sulfamidase for improved MPS IIIA brain gene therapy.

528. Liver-Mediated Gene Therapy with an Engineered Secretable GAA Transgene Results in Whole-Body Correction of Pompe Disease

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Pompe disease is a severe neuromuscular disorder caused by mutations in the lysosomal enzyme acid-alpha glucosidase (GAA), which result in the pathological accumulation of glycogen in all tissues. Enzyme replacement therapy (ERT) is available for Pompe disease, however it has only limited efficacy, high immunogenicity, and fails to correct nervous tissue and muscle fibers refractory to cross-correction. Using bioinformatics analysis and protein engineering, we developed secretable GAA transgenes for enhanced cross-correction of Pompe disease via adeno-associated virus (AAV) vector liver gene transfer. Gaa-/- mice were treated with AAV vectors optimized for hepatic expression of secretable GAA transgenes and followed for up to 10 months post-gene transfer. The AAV encoding for the most effective GAA transgene was also tested in non-human primates (NHP) in vivo and in primary human hepatocytes in vitro. Liver directed gene transfer resulted in dose- and time- dependent whole-body correction of glycogen accumulation in muscle, central nervous system, and spinal cord. The biochemical correction was accompanied by a complete normalization of cardiac hypertrophy, muscle and respiratory function, and survival undistinguishable from wild-type littermates. In these experiments, engineered secretable GAA transgenes showed superior therapeutic efficacy and markedly lower immunogenicity compared with their native GAA counterpart. Scale up to NHP, and modeling of GAA expression in primary hepatocytes, demonstrated the therapeutic potential of AAV vector-mediated liver expression of secretable GAA transgenes. Overall these findings demonstrate the therapeutic potential and support the clinical translation of this gene therapy approach for the treatment of Pompe disease.

529. Correction of Neurological Manifestations of Mucopolysaccharidosis IIIC by a Novel Rationally Designed Neurotropic AAV Gene Therapy Vector

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Mucopolysaccharidosis (MPS) type IIIC is a neurodegenerative lysosomal storage disorder caused by the lack of the heparan sulphate (HS) degrading enzyme heparan sulfate acetyl-CoA:α-glucosaminide N-acetyltransferase (HGSNAT). HGSNAT deficiency results in widespread central nervous system pathology, behavioural problems, developmental delays, sleep disturbances & dementia. HGSNAT is a transmembrane protein; therefore secretion and cross-correction are unlikely, making the development of therapies challenging. Intracranial injection of AAV could potentially restore brain enzyme levels correcting neuropathology in MPSIIIC patients. A novel AAV serotype (AAV-TT) was engineered to include key residues found in natural variants of AAV2, resulting in a gene therapy vector with extraordinary transduction characteristics in the CNS. We compared GFP expression of AAV9, Rh10 and AAV-TT in the brains of mice, demonstrating improved distribution of AAV-TT-GFP in the brain over AAV9-GFP and Rh10-GFP. AAV-TT-GFP specifically transduces neurons in the adult mouse brain. We subsequently compared the therapeutic efficacy of AAV expressing the HGSNAT transgene using the two best serotypes AAV9-HGSNAT and AAV-TT-HGSNAT in a long-term study in MPSIIIC mice, delivered via bilateral intracranial injections. At 4 months post injection, behaviour was corrected in AAV-TT-HGSNAT treated MPSIIIC mice over AAV9-HGSNAT. At 6 months post injection, both serotypes restored HGSNAT enzyme activity levels in the brain. Primary storage of total HS was decreased in the brains of AAV-TT-HGSNAT treated mice over AAV9-HGSNAT, whilst secondary storage of GM gangliosides was corrected by both. Immunohistochemical analysis of the brain showed a reduction of inflammation in AAV-TT-HGSNAT but not AAV9-HGSNAT treated mice. No anti-AAV IgG antibodies were detected against either serotype in all treated mice. Our findings demonstrate that AAV-TT is better distributed within the brain and corrects the mouse model of MPSIIIC more effectively than AAV9. This is an improved vector design for diseases with global neuropathology such as MPSIIIC.

530. Systemic Messenger RNA (mRNA) Therapy Delivered via Lipid Nanoparticle as a Treatment for MUT Class Methylmalonic Acidemia (MMA)

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Isolated methylmalonic acidemia (MMA) is a severe autosomal recessive metabolic disorder most commonly caused by mutations in methylmalonyl-CoA mutase (MUT), a mitochondrial enzyme that catalyzes the isomerization of methylmalonyl-CoA to the Krebs cycle intermediate succinyl-CoA. Despite advancement in the understanding of disease pathophysiology, MMA is mainly managed by restriction of protein in the diet, augmented by cofactor supplementation and vigilant monitoring. Even with these interventions patients continue to experience early mortality and substantial morbidity. Liver transplantation, kidney transplantation, or combined liver/kidney transplantation are the only effective treatment options, but are limited by the availability of donors. Previous studies have shown that both ubiquitous and liver-directed gene therapy using AAV8 or AAV9 vectors to deliver the *MUT* cDNA were highly effective at correcting the neonatal lethal phenotype associated with MMA in *Mut* knockout mice. These studies provided evidence that gene therapy may have clinical utility in the treatment of MMA, however limitations related to the adeno-associated viral vector, including pre-existing AAV immunity and potential genotoxicity, present hurdles for further clinical development. Alternative classes of vectors such as lipid nanoparticles (LNP) could allow for hepatic expression of MUT while circumventing barriers associated with the immune response, age and possible untoward vector genotoxicity. We are currently exploring a new class of therapy for MMA by LNP delivery of human MUT (*hMUT*) mRNA to produce enzymatically active MUT in the liver. Human fibroblasts from MMA patients with MUT deficiency transfected with *hMUT* mRNA demonstrated mitochondrial localization and enzymatic activity of hMUT. To model the hepato-, renal and cerebral manifestations of MMA, we have generated several lines of transgenic/knock-out mice to create viable animal models of MMA. $Mut^{-/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$ mice express wild-type Mut in the skeletal muscle under the control of the murine creatine kinase promoter and display severe biochemical perturbations, while mice that constitutively express the mouse orthologue of a well-characterized partial activity mutation $Mut^{-/-}$;$Tg^{CBA\text{-}Mut\text{-}p.G715V}$ manifest biochemically but are less clinically affected. Following a single intravenous administration of *hMUT* mRNA, MMA mice exhibited hepatic expression of functional hMUT protein, decreased plasma methylmalonic acid levels and increased hepatic hMUT activity. In $Mut^{-/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$ mice, 1-C-13 propionic acid oxidation increased to levels near those seen in controls three days post-administration. Finally, treatment at birth with *hMUT* mRNA was able to rescue a neonatal lethal mouse model MMA that replicates the most severe form of disease. Our data broadly demonstrate the potential of systemic mRNA replacement therapy for MUT MMA that could be used across the full spectrum of disease severity.

531. In Vivo Reprogramming of Pancreatic Duct Cells into Monohormonal Insulin Secreting β-Like Cells

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Direct reprogramming offers the possibility to instructively convert differentiated, abundant cells into other cell types for cell replacement therapy. This is usually achieved through forced activation or repression of lineage defining factors or pathways. In particular, reprogramming towards pancreatic endocrine β cells has been of great interest to diabetes research. It has been suggested that cells from various endoderm lineages can be converted to β-like cells. However, it is not clear whether cells from different lineages have similar reprogramming potential and how close induced cells resemble endogenous pancreatic β cells. Here, we report reprogramming of pancreatic duct cells through an intra-ductal delivery approach of the transcription factors Pdx1, MafA and Ngn3 in an adenoviral vector. Using this method, we observed correction of diabetes in both chemically and genetically induced diabetes models. The induced β cells were mono-hormonal, expressed genes essential for β cell function and showed glucose responsiveness. Compared to hepatic ducts reprogrammed with the same vector, pancreatic ducts demonstrated more rapid activation of β cell transcription factors and repression of markers of the cells of origin. This approach may be readily translatable to humans through the commonly performed procedure, endoscopic retrograde cholangio-pancreatography (ERCP) and provides potential for development of cell replacement therapy for type 1 diabetes patients.

532. Molecular Therapy of Melanocortin-4-Receptor Obesity by an Autoregulatory BDNF Vector

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Obesity is a major pandemic affecting 35.7% of the U.S. and increases the risk for cardiovascular disease and cancer. Mutations in the melanocortin-4-receptor (*MC4R*) comprise the most common monogenic form of severe early-onset obesity, and conventional treatments for these patients are either ineffective long-term or contraindicated. Immediately downstream of MC4R- in the key pathway for the regulation of energy balance- is brain derived neurotropic factor (BDNF). Recent genome-wide association studies have found that BDNF is one of 18 genetic loci associated with body mass index. BDNF deficiency is associated with the obesity syndromes seen in the Prader-Willi syndrome, WAGR syndrome, and *MC4R* mutations. Our previous preclinical data show that recombinant adeno-associated viral (AAV) vector-mediated BDNF gene transfer

in the hypothalamus efficiently alleviates obesity and diabetes in both diet-induced and genetic models. To facilitate clinical translation we have developed a built-in autoregulatory system to control therapeutic gene expression mimicking the body's natural feedback systems. This autoregulatory approach leads to a sustainable plateau of body weight after substantial weight loss is achieved. To further develop the targeted molecular therapy for *MC4R* obesity, we examined the efficacy and safety of autoregulatory BDNF gene therapy in *Mc4r* heterozygous mice, a model that best resembles *MC4R* obese patients. 3-month-old female *Mc4r*$^{+/-}$ mice were injected bilaterally with either 10^{10} viral particles of AAV-*Bdnf* or AAV-YFP control and monitored for 30 weeks. BDNF gene therapy prevented the development of obesity and metabolic syndromes in *Mc4r*$^{+/-}$ mice. BDNF treatment decreased body weight, decreased adiposity as measured by echoMRI, improved glucose tolerance and insulin tolerance, and increased energy expenditure as measured by indirect calorimetry. The metabolic improvements of BDNF gene therapy were not associated with adverse cardiovascular function as tail cuff manometry revealed no significant differences in systolic or diastolic blood pressure between groups. In addition, mice were subjected to a battery of tests to assess behavioral side effects including anxiety and depression, home cage monitoring, and circadian activity. No significant behavioral disturbance was observed in BDNF-treated mice. Future directions include histological processing of various organs to assess inflammation, fat deposition, bone density, or viral targeting. The same experiments will be performed on male cohorts with an additional assessment of aggression. The safety and efficacy data from these mouse studies will provide preclinical evidence that BDNF gene therapy is a viable treatment option for *MC4R*-deficient obese patients.

Preclinical Progress Towards Therapies for Neurologic Disorders

533. Life-Long Transgene Expressions of Dopamine-Synthesizing Enzymes in a Primate Model of Parkinson Disease

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The cardinal motor symptoms of Parkinson disease (PD) are associated with severe loss of dopamine in the striatum. One current strategy for gene therapy for PD involves local production of dopamine in the striatum that is achieved by inducing the expression of enzymes involved in the biosynthetic pathway for dopamine. Three enzymes are necessary for efficient biosynthesis of dopamine, namely tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase (AADC), and guanosine triphosphate cyclohydrolase I (GCH). TH converts L-tyrosine to levodopa, and then AADC converts levodopa to dopamine. GCH is the rate-limiting enzyme in the biosynthesis of tetrahydrobiopterine, a co-factor of TH activity. We previously showed the efficacy of adeno-associated virus (AAV) vector-mediated gene delivery to the putamen of these dopamine-synthesizing enzymes up to 10 months after the procedure in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity-based primate model of PD. In this study, we extend our previous findings by demonstrating behavioral recovery and a stable transgene expression in a monkey followed up for 15 years after gene therapy. The Parkinsonian monkey was received stereotaxic injections of AAV-TH, AAV-AADC, and AAV-GCH (1.5×10^{11} genome copies of each vector) in the left putamen. Two weeks after gene therapy, the monkey showed behavioral recovery in the right-side limb, which remained unchanged for 15 years and 5 months until euthanasia was carried out owing to the onset of senility. Immunohistochemistry of the post mortem brain revealed persistent expressions of the TH, AADC, and GCH genes in the lesioned putamen. Transduced neurons were broadly distributed, with the estimated transduction region occupying 91% of the left postcommissural putamen. Most of the AADC-immunoreactive (IR) or GCH-IR cells were also positive for TH. No signs of cytotoxicity nor Lewy body pathology were observed in the AAV vector-injected putamen. Although the implications should be limited because only one animal was assessed, this study provides further support that our triple transduction strategy is safe and effective, and suggesting that life-long transgene expression is possible in gene therapy for PD.

534. Second-Generation MECP2 Gene Therapy Extends Survival in a Mouse Model of Rett Syndrome (RTT) without Apparent Toxicity

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RTT is a neurodevelopmental disorder that is almost exclusively diagnosed in females and is caused by loss-of-function mutations in the X-linked transcription regulator methyl CpG-binding protein 2 (MeCP2). Published work has shown that intravenous (IV) delivery of a self-complementary 1st-generation vector (AAV9/*hMECP2*(v1)) extends the lifespan of *Mecp2*$^{-/y}$ mice. Because IV treatment was accompanied by acute liver toxicity, we decided to evaluate this vector after direct administration into the cerebrospinal fluid. As expected, administration of AAV9/*hMECP2*(v1) into the cisterna magna (ICM; 1 x 10^{12} vg/mouse) biased transgene expression away from the periphery. Although administration of AAV9/*hMECP2* (1 x 10^{12} vg, ICM) significantly extended the survival of *Mecp2*$^{-/y}$ mice, this treatment aggravated behavioral scores for hindlimb clasping and abnormal gait in *Mecp2*$^{-/y}$ mice. In WT mice, similar treatment

induced over-expression related toxicity in the form of hindlimb clasping, abnormal gait, weight loss, and reduced survival. Therefore, we proceeded to develop a safer 2nd-generation viral genome (AAV9/*hMECP2*(v2)). This 2nd-generation vector includes more regulatory elements from the endogenous MeCP2 promoter and 3' untranslated region (UTR). Notably, ICM administration of AAV9/*hMECP2*(v2) (1 x 10^{10} - 1 x 10^{11} vg/mouse) extended the survival of *Mecp2*$^{-/y}$ mice after treatment. In addition, 1 x 10^{10} vg of AAV9/*hMECP2*(v2) rescued low body weight in *Mecp2*$^{-/y}$ mice (without decreasing WT weight), and did not aggravate limb clasping or abnormal gait scores in *Mecp2*$^{-/y}$ or WT mice. Biodistribution and gene expression data revealed that transgene expression in *Mecp2*$^{-/y}$ mice was more tightly regulated by AAV9/*hMECP2*(v2) (compared to that of AAV9/*hMECP2*(v1)). In summary, this research identifies serious limitations of the previously published viral vector AAV9/*hMECP2*(v1), but begins to address those limitations through the development of a relatively safer *hMECP2*(v2) construct, which can extend *Mecp2*$^{-/y}$ lifespan at a 100-fold lower dose.

535. Expanding the CNS Transduction Profile of AAV4 by Structure-Guided Evolution

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Intracranial administration of adeno-associated viral (AAV) vectors into the cerebrospinal fluid (CSF) has enabled therapeutic gene transfer in preclinical animal models of Alzheimer, Batten, Sandhoff and other such central nervous system disorders. While certain AAV serotypes show minimal parenchymal spread with predominantly ependymal transduction (e.g., AAV2, AAV4), others show widespread CNS transduction often accompanied by systemic leakage (e.g., AAV9, AAVrh.10). Our lab previously demonstrated that the CNS transduction profile of AAV2 can be significantly enhanced by engrafting the AAV9 galactose footprint onto the capsid (the new variant AAV2g9). Here, we expand the restricted CNS transduction profile of the antigenically distinct serotype, AAV4, through structure-guided evolution in a viral encephalitis model. We engineered six different AAV4-based libraries by randomizing distinct surface loop domains on the capsid. Following intraCSF administration in mice, synthetic AAV4 variants were amplified by three rounds of evolution aided by intraperitoneal co-infection with an Adenoviral strain that induces murine encephalitis. Variants derived from two surface domains at the three fold symmetry axes were dominantly selected from the mixture of six capsid libraries. The AAV4 variants showed a range of transduction profiles ranging from restricted expression in the ependyma and choroid plexus to an improved ability to spread across the parenchyma. One variant AAV419 showed increased transduction efficiency along the rostro-caudal axis with a striking shift in tropism to neurons in the cortex, hippocampus and olfactory bulb. The expanded CNS transduction profile of these novel variants appears to inversely correlate with viral infectivity in cell culture. In addition to being able to evade neutralizing antibodies generated by natural exposure or prior vector administration, AAV419 demonstrates the feasibility of reengineering the CNS transduction profile of natural AAV isolates and is a promising vector for expression of secreted therapeutic proteins in the brain.

536. AAV5-miHTT Gene Therapy Demonstrates Broad Vector Distribution and Strong Mutant Huntingtin Lowering in a Huntington's Disease Minipig Model

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Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a CAG trinucleotide repeat expansion in the *HTT* gene. This CAG repeat expansion results in an expanded polyglutamine repeat in the huntingtin protein, causing toxic gain-of-function and affecting numerous cellular processes. Lowering the expression of mutant huntingtin and thereby reducing downstream toxic effects is hypothesized to be therapeutically beneficial. We previously showed strong reductions in huntingtin and prevention of neuronal dysfunction in HD rodent models using gene transfer of a cassette encoding microRNA targeting human huntingtin (miHTT), delivered via adeno-associated viral vector serotype 5 (AAV5-miHTT). Here, we investigated the feasibility, efficacy, and safety of AAV5-miHTT for the first time in HD animals with a larger brain size. AAV5-miHTT was administered bilaterally into the striatum and thalamus of transgenic HD (tgHD) minipigs using convection enhanced delivery. In total 24 animals were injected with 1E+13 and 3E+13 genome copies AAV5-miHTT, AAV5-GFP, or formulation buffer and followed for three months. We detected a widespread dose-dependent distribution of the vector throughout the minipig brain that correlated strongly with the miHTT expression (Pearson r = 0.9371). Expression of mutant HTT mRNA was significantly reduced by 50 to 80% in all regions transduced by AAV5-miHTT and up to 40% in the cortex, compared with control. In accordance with the intra-parenchymal HTT mRNA reduction, longitudinal ultrasensitive single-molecule counting of soluble mutant huntingtin showed significantly reduced levels of mutant huntingtin in the cerebrospinal fluid (CSF) of tgHD minipigs after AAV5-miHTT treatment. Both the surgical procedure and AAV5-miHTT treatment were well tolerated with no adverse events. Longitudinal cytokine profiles in CSF of tgHD minipigs demonstrated a consistent pattern with a transient mild increase in cytokine levels at 7 and 14 days post AAV5-miHTT injection. Immunohistochemical analysis showed a dose-dependent local microglial activation with no histological pathology in the brain of tgHD minipigs treated with the 1E+13 genome copies of AAV5-miHTT. The present study is the first demonstration of successful mutant HTT-lowering in a large animal model. The combination of widespread vector distribution, extensive huntingtin lowering, long-term expression and tolerability observed with AAV5-miHTT support further investigation of efficacy and safety in this model, the tgHD minipig, and continued preclinical development of HTT-lowering gene therapy for HD.

537. Intrathecal scAAV9 Gene Therapy Is an Effective Treatment for PPT1-Deficiency in the Preclinical INCL Mouse Model

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Infantile neuronal ceroid lipofuscinosis (INCL), or Infantile Batten disease, is a severe neurodegenerative disease caused by the deficiency of the soluble lysosomal enzyme Palmitoyl-Protein-Thioesterase-1 (PPT1), encoded by the *CLN1* gene. The pathological hallmark is the accumulation of autofluorescent storage material (AFSM) in lysosomes of most tissues, and extensive astrogliosis, neuroinflammation and neurodegeneration within the central nervous system (CNS), including the spinal cord. The onset of symptoms in humans occurs between 6 to 24 months of age, with progressive visual failure, cognitive and motor decline, seizures and premature death around 7 years of age. There is currently no cure; only palliative care. INCL mice (*CLN1*-KO) recapitulate the major features of the human disease, with extensive astrogliosis and accumulation of AFSM by 3 months, neurological deficits appearing by 4.5 months of age, and premature death occurring around 8 months. While previous neonatal gene transfer experiments using Adeno-Associated Virus (AAV) have shown some success in extending the survival of the INCL mice, these approaches have not been directly translatable, and there has been little to no improvement of their performance in behavioral testing or in their quality of life. We engineered a self-complementary AAV serotype 9 (scAAV9) encoding the human *CLN1* gene and examined survival and correction of behavioral deficits in the INCL mouse model following a single intrathecal (IT) injection of scAAV9/hCLN1 across different ages: 1 week (modeling dosing soon after birth in patients), 1 month (pre-symptomatic), 4.5 months (early-symptomatic) or 6 months of age (symptomatic). In all cases, treatment resulted in sustained supraphysiological levels of active PPT1 in the serum for up to 3 months post-dosing (longest time point examined). Treatment after onset of symptoms (>4.5 months-old) at this initial low dose showed little to no therapeutic benefit, but those treated before symptoms at 1 month of age survived approximately twice their expected lifespan. Ongoing studies also demonstrate more substantial benefits in mice treated even younger, at 1 week old. These earliest-treated mice had delayed onset of motor deficits around 15 months, and had a median survival of > 18 months. We subsequently tested a 10-fold higher dose: Mice treated at 1 month old were indistinguishable from heterozygous mice when tested at 9 months old, in terms of survival as well as motor and behavioral deficits. Treatment at the early-symptomatic stage (4.5 months) extended lifespan by 4 months and delayed the onset of motor deficits by 3.5 months. Treatment at 6 months of age extended lifespan by 1.5 months will little improvement in behavior. Our findings suggest this approach could be advanced as a treatment for patients with INCL, similar in approach to ongoing intrathecal AAV9 clinical trials for Giant Axonal Neuropathy and NCL type 6. Further, for INCL we demonstrate the importance of early intervention for maximum therapeutic benefit.

538. Silencing *SOD1* to Treat Amyotrophic Lateral Sclerosis: Preclinical Studies in Cynomolgus Monkeys

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Amyotrophic lateral sclerosis (ALS) is a devastating, invariably fatal neurological disease caused by degeneration of the motor neuron system leading to often rapidly progressing paralysis. Survival in ALS is typically 3-5 years; no treatment extends patient survival by more than three months. Approximately 20% of familial ALS and 1-3% of sporadic ALS patients carry a mutation in the gene encoding superoxide dismutase 1 (*SOD1*). Gene silencing therefore appears as a potential strategy to treat this gain-of-function disorder. Here, we selected to silence *SOD1* using an artificial miRNA that targets all mutations, and to use rAAVrh10 for delivery, given its demonstrated safety profile in CNS clinical trials. We previously established proof-of-concept in adult $SOD1^{G93A}$ mice, demonstrating that *SOD1* silencing profoundly delays disease onset and death, and significantly preserved muscle strength and motor and respiratory functions (1). We further documented that intrathecal delivery of the candidate in marmoset monkeys (*Callithrix jacchus*) significantly and safely silenced *SOD1* in lower motor neurons (1). Subsequently, one study in African green monkeys (*Chlorocebus sabaeus*) revealed that direct intrathecal injection was not optimal for vector delivery into larger nonhuman primates. The delivery protocol was therefore optimized in cynomolgus monkeys (*Macaca fascicularis*), including preimplantation of the catheter and placement of the subject at a 30° angle during delivery by infusion. Results from two studies in cynomolgus monkeys with this optimized delivery protocol will be presented. In the first study, GFP was included in the transgene to test the delivery itself. Overall, results demonstrate efficient delivery that is very reproducible. Additionally, three promoters were compared in terms of silencing efficacy (pol II chicken beta-actin CB, and pol III H1 and U6) and the CB and H1 promoters were selected for further development. However, a mild, transient elevation in liver transaminases was observed, which could not conclusively be connected to either the presence of GFP or a potential RNAi-related toxicity. In the second study, clinical constructs devoid of GFP were tested for safety and efficacy, and the CB and H1 promoters were compared. Stable liver transaminases in this study indicated that the GFP was most likely the cause of the elevation observed in the first study. Overall, the results support the view that this gene therapy candidate is safe, presents the potential to make a difference in the progression of the disease, and therefore merits further development for the treatment of *SOD1*-linked ALS in humans. (1) Borel, F. et al. Therapeutic rAAVrh10 Mediated SOD1 Silencing in Adult SOD1(G93A) Mice and Nonhuman Primates. Hum Gene Ther 27, 19-31, doi:10.1089/hum.2015.122 (2016).

539. Long Term Survival of Sheep with Tay-Sachs Disease After Delivery of a Novel Bicistronic AAV Gene Therapy Vector

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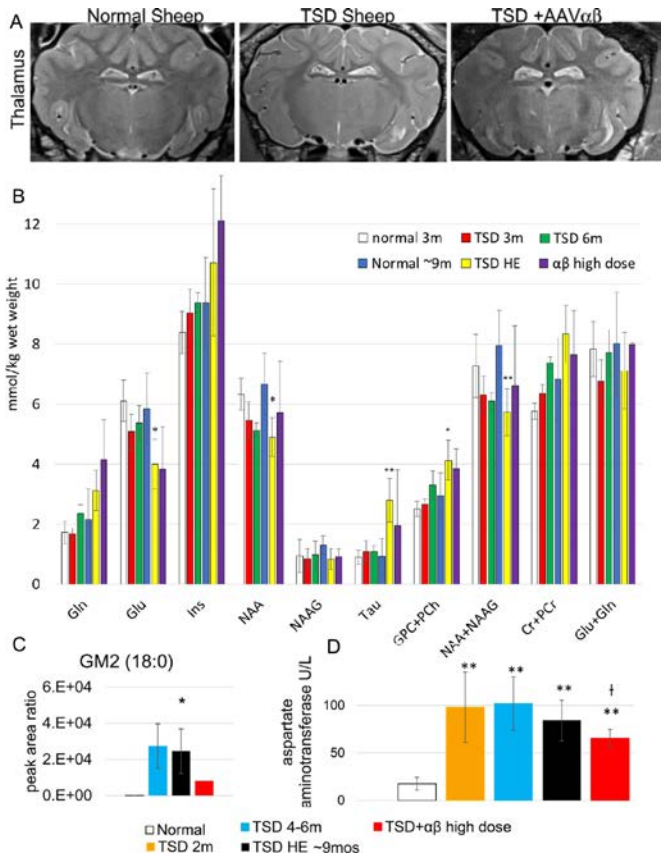
Sandhoff (SD) and Tay-Sachs disease (TSD) are fatal neurologic diseases of children caused by a deficiency of the heterodimeric lysosomal enzyme Hexosaminidase (Hex). At present, there is no efficacious FDA approved therapy for these diseases, but preclinical adeno-associated viral (AAV) gene therapy experiments in mouse and feline models of SD and a sheep model of TSD are promising. One of the therapeutic challenges of Tay-Sachs and Sandhoff disease is the requirement for simultaneous delivery of the genes for both Hex subunits (α and β). We developed a dual gene construct that results in simultaneous expression of both subunits from a single vector. TSD sheep were treated at 2-3 months of age by thalamic and lateral ventricle injection of 6×1012 v.g. of an AAVrh8 vector encoding the α and β subunits. AAV treated TSD sheep survival was increased to 17.5 ± 2.9 months compared to 9.2 ± 1.1 months for untreated TSD sheep (p=0.014). MRI and MR spectroscopy show neurodegenerative disease-specific alterations that are partly normalized by gene therapy. Hex expression was increased above untreated in all brain blocks, ranging from 0.65 fold normal at the thalamic injection to 0.05 fold normal in the cerebellum. Spinal cord Hex activity was ~0.15 fold normal, and no increase in Hex activity was detectable in CSF. Survival correlated highly with Hex activity in the frontal cortex (R2=0.95), thalamus (R2=0.95) and caudal cerebellum (R2=0.87). Targeted lipidomics of CSF from TSD sheep showed alterations in several lipid species including gangliosides GM2 (16:0 and 18:0) and GM3, ceramide, monohexosylceramide and sphingosine. AAV gene therapy partially normalized GM2, ceramide and sphingosine levels. From these studies, we conclude that broadly distributed, low levels of AAV-generated Hex activity in the CNS are therapeutic in a large animal model of Tay-Sachs disease. Figure 1. MRI and CSF measures support efficacy and increased survival in the Tay-Sachs sheep after gene therapy. A) Ultra high field MRI (7T) shows normalization of demyelination in the TSD+AAV sheep. B) MR spectroscopy of the AAV treated TSD sheep shows partial normalization of the neuronal cell marker N-acetylaspartate (NAA), taurine and myelination markers glycerophosphocholine and phosphocholine (GPC+PCh). C) CSF GM2 ganglioside levels are normalized after gene therapy and D) cell damage marker aspartate aminotransferase (AST) is reduced in TSD+AAV CSF at humane endpoint.



540. Optimization of Mitochondrial AAV-Mediated Gene Delivery for Outer Retina and Glia

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Introduction: Mitochondria disorders are a diverse group of severe conditions from nuclear and mitochondrial DNA mutations. Growing evidence suggests that mitochondrial dysfunctions are involved in both age-related and inherited retinal neurodegenerative diseases. Gene therapy has the potential to treat such diseases but efficient mitochondrial targeting remains a challenge in the field. In this study, we designed and optimized AAV-mediated gene delivery for targeting mitochondria in photoreceptors and Müller glia. We aim to determine whether a nuclear-encoded transgene redirected to the mitochondria or a mito-targeted AAV capsid encoding a mitochondrial transgene is a more selective and/or efficient approach.

Methods: Computational methods were used to predict cleavable N-terminal mitochondrial targeting signal (MTS) pre-sequences that redirect selectively translated proteins from the cytoplasm to the mitochondria. The MTS sequences were cloned on the N-terminal of tdTomato to create fusion fluorescent transgenes and transfected in HEK293T cells. Immunochemistry, RT-qPCR, and western blot data

were collected to quantify and analyze the efficiency/selectivity of each MTS. We then engineered two different AAV-mediated delivery systems to target mitochondria in photoreceptors and glia: 1) A nuclear-encoded MTS-tdTomato under the control of a CAG promoter packaged with retinal-specific AAV variants. 2) A mitochondrial-encoded tdTomato under the control of a mitochondrial promoter (HSP) packaged into mito-targeted AAVs. MTS-VP2 fusion protein was supplied *in trans* during packaging for selective recognition and transport of viral capsids to the mitochondria. All rAAV vectors were injected intravitreally into C57BL/6 mice. Animals were euthanized 3 weeks post-injections and retinas were collected for further analysis.

Results: We identified MTS sequences with different levels of selectivity for redirecting transgenes to the mitochondria *in vitro*. The Ornithine Transcarbamylase (OTC) pre-sequence was the most selective for redirecting the transgene to the mitochondria while others, like COX8A, exhibited higher levels of nuclear expression. Preliminary *in vivo* results showed that MTS sequences with very selective mitochondrial tropisms negatively impacts the packaging of mito-targeted AAV capsids, redirecting the VP2 subunit to the mitochondria before proper capsid formation. MTS sequences with both nuclear and mitochondrial tropisms are better fitted for AAV packaging. Viral vectors re-directing proteins from nuclear-encoded transgenes allowed cell-specific tropism but require mitochondria-selective MTS sequences such as OTC to avoid nuclear off-targeting.

Conclusion: The choice of the MTS sequence highly impacts both viral- and transgene-mediated mitochondrial gene delivery. Redirecting the capsid towards the mitochondria is the most selective approach, however it does not allow yet for particular cell tropisms. There is a great need for the characterization and optimization of both methods to achieve cell-specific and mitochondrial selective targeting. Based on these results we will be able to design better gene therapies to treat animal models of mitochondrial disease and move research forward to clinical applications.

Preclinical Optimization of Immunotherapies

541. Constitutive Signaling from an Engineered IL-7 Receptor Promotes Durable Tumor Elimination by Tumor Redirected T-Cells

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A lack of efficacy against solid tumors remains a major challenge for adoptive T-cell immune therapy. Failure of T-cells to expand and persist is likely due to a combination of the immunosuppressive ligands produced by the solid tumor and lack of effective T-cell activation in vivo. Provision of Signal 1 (T-cell receptor activation), Signal 2 (co-stimulation), and Signal 3 (cytokines) is necessary for optimal T-cell activity, but the full complement of these signals is rarely available within the tumor microenvironment. While modification of T-cells to express chimeric antigen receptors (CARs) can provide Signals 1 and 2 upon antigen ligation, CAR ligation does not induce sufficient Signal 3 to sustain T-cell expansion in vivo. Systemic cytokine administration, or secretion of cytokines by T-cells themselves, have not achieved desired clinical effectiveness and also demonstrated serious adverse effects, We aimed to restore Signal 3 selectively to CAR T-cells by co-expression of an IL7 receptor rendered constitutively active by a homodimerizing transmembrane domain. To avert homeostatic disruption, IL-7 binding by the receptor was abolished by replacement of its ectodomain with that of CD34, generating a chimeric IL7 receptor (C7R). C7R activated STAT5 signaling in both CD4 and CD8 T-cells and prolonged T-cell persistence in conditions devoid of antigen or external cytokines, without leading to sustained autonomous growth. During repeat co-cultures with tumor cells, however, C7R-expressing GD2-CAR (C7R-GD2-CAR) T-cells maintained antigen-directed expansion and cytotoxicity, whereas GD2-CAR T-cells alone contracted and lost their ability to eliminate tumors. This was associated with a significant reduction of apoptosis in C7R-GD2-CAR T-cells (mean 40%) compared to GD2-CAR T-cells. C7R also enhanced GD2-CAR T-cell treatment of 1-week-old xenograft LAN-1 neuroblastomas in NSG mice. $1x10^6$ intravenously injected C7R-GD2-CAR T-cells were able to eradicate tumors, in contrast to marginal tumor control by GD2-CAR T-cells. Similar results were obtained when we evaluated C7R-GD2-CAR T-cells in a xenograft metastatic tumor model using the CHLA-255 neuroblastoma cell line. We also tested the ability of C7R to enhance EphA2-CAR T-cells in an intracranial xenograft U373 glioblastoma tumor model in SCID mice. Bioluminescent tumor imaging revealed that at a dose of 10^4 T-cells, EphA2-CAR T-cells could not control tumor outgrowth, whereas C7R-EphA2-CAR T-cells cleared tumors in all mice. Median survival of control and EphA2-CAR T-cell treated mice was 41 days after tumor engraftment, while mice receiving C7R-EphA2-CAR T-cells remained tumor free at Day 112 when the experiment was terminated. Although C7R-GD2-CAR T-cells do not expand or persist long-term in the absence of antigen stimulation/co-stimulation, co-expression of the iC9 safety switch does not attenuate their antitumor activity and therefore provides another level of protection against autonomous growth of C7R modified T cells. In summary, providing Signal 3 to T-cells through C7R enhances the persistence and anti-tumor efficacy of CAR T-cells across multiple tumor models, and should not non-specifically activate bystander cells.

542. Transgenic c-MPL Provides Ligand-Dependent Co-Stimulation and Cytokine Signals to TCR-Engineered T Cells

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Engineering polyclonal T cells with a transgenic T cell receptor (TCR) can efficiently redirect antigen-specificity to tumor-associated antigens. Co-stimulation (signal 2) and cytokine signals (signal 3) that

are essential for full and sustained T cell activation, however, rely on the host environment and are often impaired in cancer patients. We therefore explored whether incorporation of a single additional gene-modification could provide both signals 2 and 3 in TCR-transgenic T cells and thereby increase anti-tumor activity and persistence after adoptive transfer. The non-lymphoid hematopoietic growth factor receptor c-MPL (myeloproliferative leukemia), which is the receptor for thrombopoietin (TPO), activates signaling pathways that overlap with conventional co-stimulatory and cytokine pathways. TPO is present systemically and locally in the bone marrow environment of patients with hematologic malignancies, and c-MPL agonist drugs are available clinically. Thus, we hypothesized that c-MPL+ T cells can receive agonist stimulation systemically, locally in the tumor microenvironment or pharmacologically with a c-MPL agonist drug. We generated a retroviral vector to express c-MPL in activated T cells. Transduction of polyclonal activated T cells was highly efficient in both CD4+ and CD8+ T cells. T cells expanded and proliferated in response to TPO in vitro, and persisted longer after adoptive transfer in immunodeficient human TPO transgenic mice under steady-state conditions. When c-MPL was co-expressed with a tumor-specific TCR targeting survivin, we observed improved T cell expansion, and enhanced tumor cell killing and cytokine production in c-MPL+ transgenic T cells. These effects were associated with improved immune synapse formation and preservation of a central memory phenotype of the T cells. Gene expression studies identified activation of the type I interferon pathway as a novel pathway by which c-MPL mediates co-stimulation in T cells. In vivo in a leukemia xenograft model, pharmacologic c-MPL activation led to improved anti-tumor activity. In conclusion, we describe a novel immunotherapeutic strategy that incorporates both co-stimulation and cytokine signals via one genetic modification to tumor-targeted TCR- transgenic T cells and thus enhances anti-tumor activity. Additionally, we identify activation of the type I Interferon signaling pathway as a molecular mechanism that contributes to c-MPL ligand-dependent co-stimulation in engineered T cells.

543. Long Term Persistence, Location in Secondary Lymphoid Tissues and Phenotype of CD19 CAR-T Cells in Mice

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CD19 CAR-T cells are effective in treating B cell malignancies in patients and can persist and cause B cell depletion. However, studies of their location and phenotype in humans have largely been limited to analysis of easily accessible sites such as bone marrow and blood. We introduced the Strep-tag II sequence into murine CD19 4-1BB/CD3ζ and CD28/CD3ζ CARs to provide a marker for identifying CAR-T cell and analyzing the level of CAR expression in tissue sites in vivo [Liu et al., Nature Biotech, 2016]. We administered murine T cells modified with STII CARs and tracked their migration, phenotype, the level of CAR expression, and function in vivo. $CD4^+$ and $CD8^+$ CAR-T cells expanded after infusion and induced long term B-cell aplasia. We detected persistent populations in blood, bone marrow, spleen, and lymph nodes for >14 months after CAR-T cell infusion. The 4-1BB/CD3ζ CAR resulted in higher levels of persisting $CD8^+$ CAR-T cells in blood, spleen and lymph nodes compared with the CD28/ CD3ζ CAR, but levels of $CD4^+$ CAR-T cells at these sites were similar. At 1, 2, 6 months after therapy, 4-1BB/CD3ζ and CD28/CD3ζ CAR-T cells were present in the bone marrow, which is the site where the antigen is persistent, and expressed a predominantly $CD62L^-$, $PD\text{-}1^+$ phenotype and high levels of cell surface CAR, measured by staining with anti-STII mAb. At the same time points, CAR-T cells in spleen and lymph node were predominantly $CD62L^+$ $PD\text{-}1^-$ consistent with a T_{CM} phenotype, and expressed lower levels of CAR. The lower level of CAR expression on $CD62L^+$ T cells in LN and spleen was consistent with the retroviral promoter being less active in quiescent T_{CM} cells. When isolated from LN and activated with anti- CD3/CD28 mAb, the CAR-T cells upregulated expression of the CAR and were capable of recognizing $CD19^+$ target cells. To address the whether the quiescent 4-1BB/CD3ζ and CD28/CD3ζ CAR-T cells that formed T_{CM} populations expressed sufficient amounts of the CAR to recognize B cells, we isolated CAR-T cells from the spleen of mice with long term B-cell aplasia, and adoptively transferred them to secondary recipients. Transferred 4-1BB/CD3ζ and CD28/CD3ζ CAR-T induced B-cell aplasia in secondary recipients, demonstrating that these cells exhibited characteristics of functional memory cells. These data demonstrate that T cells signaled through CD19 4-1BB/CD3ζ and CD28/CD3ζ CARs can form memory populations with characteristic T_{EM} and T_{CM} phenotypes. Further studies in settings where the antigen is completely cleared are required to determine how different signaling domains in CARs affect the magnitude of memory formation and maintenance.

544. The Suppressive Solid Tumor Microenvironment Impairs the Activity of CAR-T Cells and Can Be Targeted by Chimeric Activating Receptor-Bearing Natural Killer Cells

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Background: The anti-tumor activity of chimeric antigen receptor (CAR)-bearing lymphocytes within solid tumor microenvironments (TMEs) is suppressed by myeloid-derived suppressor cells (MDSCs) that express inhibitory ligands and suppressive cytokines. In retrospective analysis of samples from a phase 1 trial at our institution, patients with neuroblastoma who received GD2 antigen-directed CAR-T cells that progressed had high numbers of MDSCs preinfusion that increased further at the time of progression. In contrast, patients who responded had no increase in MDSCs compared to normal donors. Hence, MDSCs may represent an important target in the quest to enhance CAR-T cell activity. Human MDSCs express ligands for the activating receptor, NKG2D, found on natural killer (NK) cells. **We hypothesized** that NK cells expressing a highly cytotoxic version of this activating receptor, comprising the ectodomain of NKG2D fused to CD3-zeta endodomain (NKG2D.zeta), would kill NKG2D ligandexpressing autologous MDSCs. NKG2D.zetamodified NK cells can thereby alter the TME and improve anti-tumor responses to CART cells. Methods:

We established a novel *in vivo* TME model of neuroblastoma in which human MDSCs, co-implanted with a neuroblastoma cell line, induce disordered vasculature, tumor supportive stroma, and immune suppression, resulting in enhanced tumor growth and inhibition of CAR-T cell activity. In this model, we examined the ability of NKG2D.zeta-NK cells to eliminate MDSCs, to attract tumor-specific T cells, and improve CAR-T cell function leading to tumor regression. As a clinical correlate, we examined the *ex vivo* activity of patient-derived NKG2D.zeta NK cells against autologous MDSCs from patients with neuroblastoma. Results: Human MDSCs secreted suppressive cytokines, expressed PDL1/2, and inhibited Tcell function, resulting in impaired response to CAR-T cells. Infusion of NKG2D.zeta NK cells reduced tumor burden by decreasing intra-tumoral MDSCs. MDSC reduction correlated with decreased circulating TGF-beta, and increased overall survival (median 71 days vs. 30 days in control-NK cell mice, $p = 0.01$). NKG2D.zeta NK cells increased the anti-tumor activity of subsequently infused GD2.CAR-T cells (mean 105 cubic mm tumor volume vs. 786 cubic mm tumor volume in CAR-T cell only group, $p = 0.01$) and increased overall survival (median survival 95 days vs. 50 days in CAR-T cell only group, $p = 0.003$). NKG2D.zeta NK cells generated from two patients with baseline high MDSC burdens exhibited specific killing of autologous MDSCs *ex vivo*. Significance: NKG2D.zeta NK cells reduced MDSC burden within a solid TME model, resulting in decreased immune suppression and enhanced CAR-T cell function. As proof-of-principle, NKG2D.zeta NK cells generated from patients with high MDSC burdens who failed to respond to CAR-T cells mediated killing of autologous MDSCs. Our results provide a rationale for a novel combination therapy utilizing the anti-TME effects of NKG2D.zeta NK cells with the antitumor activity of CAR-T cells for the treatment of solid tumors.

545. Synergistic CD4 and CD8 T Cell Receptor Platform for Stem Cell Based Therapy for Cancer

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We have been developing approaches to improve the outcome of patients with solid tumors based on novel immunotherapies to boost anti-cancer T cell responses. Re-engineering patient's mature immune cells and administering billions of these cells have shown to be promising but unfortunately, the anti-tumor effect is largely short-lived. In an effort to generate tumor-associated antigen (TAA) specific T cells we have identified NY-ESO-1 as the prototypic TAA for cancer immunotherapy. We have recently discovered a novel and distinct subset of human CD4+ Th1 cells that directly recognize NY-ESO-1 naturally presented by MHC II on cancer cells. In contrast to conventional CD4 T cells, these tumor-recognizing (TR) CD4 T cells also potently provide help to CD8 T cells in an antigen-presenting cell (APC) independent fashion. Our central hypothesis is that CD4TCR engineered human hematopoietic stem/progenitor cells (hHSC) will lead to durable in vivo supply of fully active TR-CD4 cells with anti-tumor activity, and provide sustained help to co-injected CD8TCR transduced effector T cells (which will serve to immediately "debulk" the tumor), leading to long-lasting tumor rejection. Results: We successfully finalized molecular cloning, construction and generation of lentiviral viral vectors with two unique NY-ESO-1 (TR)CD4TCRs (MHC II restricted HLA DR1 and DP4), as well as, another novel CD8 (MHC I HLA A2.1) TCR for NY-ESO-1. Transduction efficiency of hHSC was high with transgene expression levels of 40-51% (0.5-1 vector copies/cell); and 65-92% tetramer + for mature T cells. We confirmed specific functional activity of all TCRs by co-incubating transduced T cells with various tumor targets (SKMEL-37, MZ19, or aAPC: K562/DR1/DP4/A2.1 +/- cognate peptides) by ELISA and intracellular staining for IFNg. For this project we generated new transgenic mouse models based on a highly immunodeficient NSG background with expression of human MHC II DP4 or DR1 in order to study hHSC differentiation/function in vivo and to test anti-human cancer activity with our adoptive cell transfer (ACT) platform. Utilizing these transgenic mice, we injected CD34+ hHSC transduced with (TR) CD4 TCR and after 2 to 3 months we confirmed generation of engineered TCR expressing human T cells by FACS-based immunophenotyping through blood sampling. We then moved forward to test anti-tumor efficacy in vivo with s.q. injection of human melanoma (MZ19) or human ovarian carcinoma (A2780/A2/NY) cells in mice injected with (TR)CD4 TCR transduced or non-transduced-control hHSC. We injected a low dose ($5x10^5$ cells) of NY-ESO-1 HLA A2.1 CD8TCR-transduced hPBMC and followed tumor growth. Remarkable tumor control was obtained ($p<0.05$) in the mouse group that received (TR) CD4 TCR transduced hHSC (tumor size=28mm^2; SE+/-11) versus mice that received untransduced hHSC (152.7mm^2; SE+/-24) or control untreated (no hHSC, no CD8 T cell ACT) (196mm^2; SE+/-40) assessed at day 32 after tumor (A2780/A2/NY) injection.Conclusion: Here we demonstrate for the first time that a combined ACT approach of (TR) CD4 TCR transduced hHSC with CD8 TCR transduced PBMC leads to a synergistic and efficient in vivo control of tumor burden. These results provide basis to pursue a Phase I/IIa clinical trial based on our novel adoptive cell immunotherapy platform to benefit patients with advanced solid tumors.

546. Dual-Switch GoCAR-T Cells: Dual Molecular Switches to Control Activation and Elimination of CAR-T Cells to Target CD123$^+$ Cancer *In Vivo*

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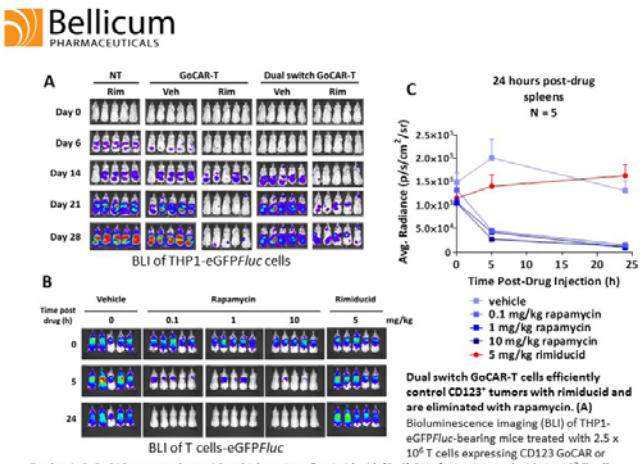
Background Improvement of chimeric antigen receptor (CAR)-T immunotherapies requires controlled activation and termination of the T cells when transfused into patients. Here we present two independently regulated molecular switches that can elicit specific and rapid induction of cellular responses upon exposure to their cognate ligands. Cell activation is controlled by the homodimerizer rimiducid that triggers signaling cascades downstream of MyD88 and CD40 via an engineered protein termed iMC. A rapamycin-controlled pro-apoptotic switch (iRC9), that induces dimerization of caspase-9 mitigates possible toxicity from excessive CAR-T function. When combined with a first generation CD123-specific CAR, these molecular switches allow for specific and efficient regulation of engineered T cells to control Acute Myelogenous Leukemia (AML) *in vitro* and *in vivo*.

Methods & Results T cells were activated and co-transduced with CD123 GoCAR (pSFG-iMC.2A-CAR.ζ) and rapamycin enabled CaspaCIDe (pSFG-iRC9.2A-ΔCD19) vectors to generate Dual-switch GoCAR-T cells. Combined transduction of the rapamycin directed CaspaCIDe and CD123 GoCAR vectors into T cells did not adversely affect the antitumor efficacy of GoCAR-T cells, which eliminated CD123$^+$ THP1 tumor cells in a co-culture assay at 1:10 effector to target ratio but not CD123$^-$ HPAC tumor cells (4.49% THP1-*eGFPfluc* cells remained in GoCAR-modified cultures treated with 1nM rimiducid versus 4.23% for Dual switch GoCAR). When challenged in a THP1-eGFP*fluc* tumor-bearing mouse model, activation of the on-switch by rimiducid treatment of mice implanted with either GoCAR or Dual switch GoCAR-T cells enhanced tumor killing (BLI) and T-cell expansion (splenocyte flow cytometry and VCN analyses). Deployment of the off-switch induced fast (½ V_{max} ~ 8 hours) and efficient elimination of T cells (Dual switch GoCAR-T = 77.6% AnnV$^+$ versus NT = 2.2% treated with 1 nM rapamycin) in a caspase-3 activation assay with real-time monitoring by the Incucyte as well as annexin V detection by flow cytometry. Importantly, the off-switch is insensitive to high rimiducid concentration (the on-switch regulator), thus avoiding cross talk between the two molecular switches. *In vivo* assessment of the suicide switch was performed with eGFP*luciferase* (eGFP*Fluc*)-labeled CD123 Dual switch GoCAR-T cells in NSG mice. Rapamycin, but not rimiducid, treatment efficiently eliminated Dual switch GoCAR-T cells within 24 hours in NSG mice, which is similar to the clinically validated rimiducid-regulated CaspaCIDe switch.

Summary: Dual switch GoCAR-T, a novel platform comprising a first generation CAR combined with regulated activation and apoptotic signaling elements, effectively controlled tumor growth and T-cell expansion and elimination *in-vitro* and *in vivo*. This dual switch technology provides a user-controlled system for managing persistence and safety of tumor antigen-specific CAR-T cells.



Dual switch GoCAR vectors along with vehicle or 1 mg/kg rimiducid. **(B, C)** BLI of mice injected with 1 x 10^7 T cells expressing CD123 Dual switch GoCAR vectors and treated with vehicle, rimiducid or rapamycin.

547. Enhancement of PSMA-Directed Car Adoptive T Cells by PD-1/PD-L1 Blockade

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Background: Chimeric antigen receptor (CAR) T-cell therapy in solid tumors has not achieved the clinical success that has been observed in hematologic malignancies. The failure of a substantial CAR-mediated T cell response in solid tumors relates to a number of factors - including CAR T cell inactivation and possible exclusion from the tumor mass, the reciprocal interactions between tumor and stromal cells, and propensity of cancer like prostate to disseminate preferentially to bone. We focused on human prostate-specific membrane antigen (hPSMA) CAR T cell targeting in prostate tumors. **Purpose:** To investigate the efficacy of human hPSMA CAR-directed T cell therapy in an appropriate animal model in the combination with anti-hPD1 mAb immune modulation therapy. **Experimental Design:** A new model to study PSMA targeting in prostate cancer was developed. Second generation anti-hPSMA human CAR T cells, expressing a Click Beetle Red luciferase reporter, were used to visualize T cell trafficking and persistence by bioluminescence imaging (BLI) - in both the presence and absence of the target antigen, and in combination with PD-1 blocking antibody. *Ex vivo* H&E and immunofluorescence (IF) imaging provided confirmatory data.**Results:** Anti-hPSMA CAR T cell monotherapy was not effective against established subcutaneous Myc-Cap:hPSMA(+) positive tumors. A Winn Assay demonstrated that anti-hPSMA CAR T cells can inhibit Myc-CaP:hPSMA(+) tumor growth, and that this inhibition is hPSMA dependent. Myc-CaP tumors are PD-L1 positive in both an immunocompromised and immunocompetent mice, and the restriction of CD3$^+$ T cells from the interior of Myc-CaP WT tumors in immunocompetent FVB/N mice was related to *PD-L1/PD1* engagement. Following anti-PD-1 treatment, the restriction of CD3$^+$ T cells was reversed, and a tumor-treatment response was observed. In adoptive CAR T cell experiments, we showed that combined treatment with anti-hPD1 antibody significantly inhibited Myc-CaP:hPSMA(+) tumor growth and that the antitumor response mediated by this combined therapy was both anti-hPD1- and hPSMA-specific. The treatment response was confirmed by H&E and TUNEL staining of the tumor 24 hours after initiation of treatment. The presence of the PMSA antigen on the tumor cells was associated with rapid clearance of the CAR T cell population, based on the intensity decrease of the bioluminescence signal (reflecting the presence and number of injected CAR T cells).**Conclusion:** We show that second generation anti-hPSMA-directed CAR T cell monotherapy of Myc-CaP:PSMA(+) tumors is suboptimal; whereas the combination of anti-hPSMA-directed CAR T cells plus anti-PD1 mAb immune modulation provides a short-duration treatment response. These results also suggest that other immune modulation mechanisms, which further reverse the restriction CAR T cell targeting and persistence in hPSMA expressing Myc-CaP tumors, are required to provide optimal CAR T cell therapy.

548. From the CART Workshop: Selecting an EphA2-CAR for the Immunotherapy of GBM

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Background: Glioblastoma (GBM) is the most aggressive primary brain tumor in humans, and is virtually incurable with conventional therapies. CAR T-cell therapy targeting the EphA2 GBM antigen is an attractive approach to improve outcomes since EphA2 is highly expressed in GBM but not in normal brain. We previously had generated an EphA2-CAR consisting of the 4H5 scFv, which recognizes a cancer-specific conformational EphA2 epitope, a CH2CH3 spacer, a transmembrane (TM) domain and a CD28.ζ endodomain. T cells expressing this CAR had potent antitumor activity in a glioma xenograft model (Chow et al, Mol Ther 2012). However, for future clinical development, CH2CH3 domains are not ideal since they bind Fc receptors. In addition, we had not defined the optimal CAR endodomain. We therefore generated a panel of EphA2-CARs with a short spacer region (SSR) that is devoid of Fc receptor binding sites, and evaluated their effector function. **Methods/Results:** We constructed retroviral vectors encoding i) a EphA2-CAR consisting of the 4H5 scFv, a SSR, a CD28 TM domain, and CD28.ζ, CD137.ζ, or CD28. CD137.ζ endodomains, ii) a 2A sequence and iii) truncated CD19 (tCD19). After transduction of CD3/CD28-activated T cells, 60-80% of cells expressed tCD19. CAR expression was confirmed by Western blot analysis. Phenotypic analysis revealed no significant differences between generated CAR T-cell lines. CAR T cells only killed EphA2+ target cells (U373, A549), secreted IFNγ and IL2 in an antigen-dependent manner, and expanded for at least 14 days after repeat stimulations with EphA2+ tumor cells. While there was some donor variability in regards to cytokine production and T-cell proliferation, no significant difference was observed between CD28.ζ, CD137.ζ, or CD28.CD137.ζ CAR T cells. *In vivo*, $2x10^6$ CD28.ζ, CD137.ζ, or CD28. CD137.ζ CAR T cells had potent antitumor activity after intratumoral injection in the U373 glioma model with 4/5, 5/5, and 4/5 mice being cured. CD28.ζ and CD137.ζ CAR T cells were tested at additional doses ($5x10^5$, $1x10^5$, $1x10^4$). CD28.ζ CAR T cells cured 3/5 mice at cell doses of $5x10^5$ and $1x10^5$, and CD137.ζ CAR T cells cured 3/5 mice at $5x10^5$ and 2/5 at $1x10^5$, respectively. At a cell dose of $1x10^4$ CD28.ζ and CD137.ζ CAR T cells only had transient antitumor activity. Comparing persistence of CD28.ζ and CD137.ζ CAR T cells after intratumoral injection *in vivo* revealed no significant difference. **Conclusions:** We demonstrate here that CD28.ζ and CD137.ζ EphA2-CAR T cells have similar effector function resulting in potent antitumor activity, and that inclusion of CD137 does not improve CD28.ζ CARs. Based on our findings, we selected CD28.ζ EphA2-CARs for future clinical development since CD28.ζ CAR T cells do not persist long term in humans. While we are targeting a cancer-specific conformational epitope, and have replaced tCD19 with an inducible suicide gene for clinical grade vector production, limited T-cell persistence should further reduce the inherent risk of 'on target/off cancer toxicity' in a 'first-in-human' clinical study.

AAV Vectors III

549. Modulating AAP Dependency on AAV Variants While Retaining *In Vivo* Infectivity

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Efforts to engineer effective gene transfer vehicles tailored to specific disease indications or that have immunological profiles distinct from natural serotypes are an active area of research. In line with these efforts, we previously generated a putative AAV phylogeny using ancestral sequence reconstruction, and have demonstrated that capsids comprising nine putative ancestral nodes form functional virions with a range of phenotypes. We harnessed these functional intermediates to gain insight into capsid assembly mechanisms, and demonstrate that requirement for the Assembly Activating Protein (AAP) in capsid assembly ranges from strict dependence to full independence across the putative ancestors and 12 natural serotypes selected such that each phylogenetic branch is represented. The AAP dependence phenotype diverges at particular nodes, such as at Anc82, the common ancestor of AAV8 (AAP-dependent) and AAV9 (partially AAP independent). We identify 12 amino acids that have shared identity within the AAP-independent branch, that have a different—yet shared—identity within the AAP-dependent branch serotypes. These residues constitute a motif that when engrafted onto the Anc82 capsid, change its AAP dependence phenotype from strictly dependent on AAP for assembly to partially independent, constituting a hybrid variant we name Anc82DI. Nine of the twelve residues lie at the VP trimer interface, and indicate potentially stronger interaction with its inter-monomeric contact residue. Anc82 and Anc82DI demonstrate a significant difference in capsid melting temperature, and Anc82DI demonstrates a 3-fold increase in production titer over Anc82.

Given these observed phenotypic changes, we sought to compare the in vivo transduction profiles of Anc82 and the DI variant. Mice were injected retro-orbitally with 1×10^{11} total vg of AAV8, Anc82, or Anc82DI capsids packaged with a CB7-hA1AT-T2A-EGFP genome. Results indicate that Anc82, the second of our putative ancestral capsids to be tested in vivo, is capable of transducing murine liver, demonstrated by appreciable levels of hA1AT in serum samples collected over the 30 day study, and EGFP-positive liver sections. Anc82DI showed an increase in transduction over its parental strain Anc82 by these measures. In some animals, Anc82DI transduction approached or even exceeded serum hA1AT levels and EGFP in liver sections in animals injected with AAV8 vectors. Our studies provide evidence that inter-monomeric interactions within the AAV capsid may impact vector phenotypes including production titers and transduction efficiency.

550. High-Throughput Screening Identifies Kinase Inhibitors That Increase Dual AAV Vectors Transduction Efficiency

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Vectors based on AAV are among the most efficient and safe for in vivo gene therapy. AAV cargo capacity limited to 5kb can be expanded to 9kb in the retina by dual AAV vectors by splitting a large transgene expression cassette in two halves independently packaged in two single AAV vectors. Their co-administration results in AAV genome intermolecular recombination and full-length transgene expression which is however greatly reduced compared to that of a single AAV. As AAV intracellular trafficking and transduction are negatively affected by phosphorylation of capsid residues, we set to identify kinase inhibitors that are able to increase dual AAV vector transduction. HEK293 cells were infected with dual AAV serotype 2 vectors expressing EGFP; cells incubated with Calpain 1 inhibitor (a proteasome inhibitor known to enhance AAV transduction, PI) were used as positive control. High throughput screening of a kinase inhibitors library using the OPERA system identified 12 positive hits. Seven of them were then confirmed by both susceptibility to a dose response curve and ability to outperform the PI induction by Western blot analysis (WB). We found that 3 compounds are more specific for AAV transduction than for plasmid transfection and, in one case, this increase is stronger for dual than for single AAV vector. As the kinase inhibitors may be promiscous, siRNA-mediated silencing of targeted kinases identified those involved in the increase of AAV transduction. The mechanism underlying this increase remains to be elucidated. Some of these compounds improve dual AAV8 transduction following subretinal delivery in mice. Our study identifies kinase inhibitors that increase dual and single AAV transduction thus highlighting pathways that may be involved in AAV post-entry steps.

551. A Robust System for Production of Superabundant VP1 Recombinant AAV Vectors

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Recombinant adeno-associated viral (rAAV) vectors have been widely used in human gene therapy. One major challenge for its broad application clinically is to produce high quality vectors in mass quantity. Here, we report an efficient and scalable suspension cell culture system for production of VP1 superabundant rAAV vectors. This system includes three major components: one cytoplasmic vaccinia carrier containing all AAV *trans* factors; one Ad/AAV hybrid vector carrying the required rAAV *cis* genomes; and a suspension cell line, QW158-7, engineered from Hela-S3 expressing the wtAd E1a and E1b proteins. All the AAV *trans* factors, Rep78, Rep52, VP1, VP2, and VP3, were stably built into one single carrier through maximal alterative codon election of Rep52 and VP1. Furthermore, a dual vaccinia promoter was developed to enhance VP2&VP3 expression. The wild type adenovirus was eliminated from the system through the generation of QW158-7 cells. The newly enhanced system can consistently produce $\sim 1 \times 10^{15}$ genome containing rAAV vectors per liter of suspension cells. Moreover, the capsid composition of rAAV vectors produced is markedly different from those from the traditional vector production system in that VP1 protein is more abundant than VP2 protein (19:1 vs 1:1). The unique VP1 superabundant rAAV vectors produced in this new system exhibited improved transduction *in vivo* after intravitreal injection. Taken together, this paper presented a new system for producing high quality rAAV vectors (higher transduction activity, no rcAAV particles), which is transfection-independent, efficient, stable, safe and easy to scale-up.

552. Several Novel Adeno-Associated Virus (AAV) Capsids Show Unique Retinal Transduction by Intravitreal Delivery to Adult C57Bl/6 Mice

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AAV delivered to the retina via the subretinal space in human patients has shown promise in treating retinal degeneration, yet delivery to the vitreous is appealing because of the benefits to retinal transduction (*e.g.*, widespread transduction, ease of delivery). While retinal ganglion cells (RGC) can be targeted by this route of administration, deeper retinal cell layers have traditionally been more difficult to transduce efficiently. Rational design studies have implicated features such as heparin sulfate proteoglycan binding or galactose proteoglycan binding as having a role in AAV retinal penetration and cellular tropism following intravitreal delivery. We propose that newer functionally distinct AAV capsids, such as those developed through directed evolution approaches, could uncover additional unique capsid features important for AAV retinal transduction. Further, the vitreous can contain neutralizing antibodies to common AAV serotypes, thereby generating a need for serologically distinct AAV capsids. Initial studies examined the retinal transduction patterns of AAV capsids from non-ocular selections, but which had unique brain tropisms in mice. These included a shuffled AAV capsid with enhanced astrocyte tropism (AstroNrF1.03) which led to high expression throughout the retina as seen by fundoscopy of GFP expression following intravitreal injection. Another shuffled capsid clone with enhanced brain transduction (ITcordNr3.03) showed low overall transduction by this route, but targeted a unique pattern of cells. To specifically generate capsids with enhanced retinal transduction, a library containing shuffled natural serotypes (AAV1, 2, 6, 8, 9), along with capsid clones recovered from previous brain-directed evolutionary approaches, was intravitreally injected into mice with labeled RGCs. Retinas were harvested and RGCs were separated from the rest of the retinal cells, creating "RGC+" and "RGC-" populations of recovered AAV clones. Compared to AAV2, one of the RGC- clones penetrated deeper into the retina, but also transduced RGCs. Together, we suggest that the unique capsids we have recovered through directed evolution

strategies, specific for the eye or otherwise, offer unique reagents for ocular gene transfer than have increased likelihood of escaping neutralizing antibodies against natural AAV serotypes.

553. Hybrid *piggyBac*/AAV-CFTRdR Viral Delivery to a Cystic Fibrosis Pig Model Corrects the Airway Phenotype *In Vivo*

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Cystic fibrosis (CF) is a common genetic disease in which loss of cystic fibrosis transmembrane conductance regulator (*CFTR*) leads to dysregulation of electrolyte transport in multiple organ systems. Though several organs are affected, lung disease is the leading cause of morbidity and mortality in people with CF. The CF pig model engineered by gene targeting recapitulates human airway disease. Gene therapy for CF has made significant advances in vector development towards the goal of a single treatment for this disease. Here, we combine a recombinant *piggyBac* transposon with an AAV vector, which upon airway delivery can integrate into the host genome, for potentially life-long CFTR expression. This hybrid *piggyBac*/AAV carries a *CFTR* cDNA mini-gene lacking a portion of the R-domain (dR) to accommodate the carrying capacity constraints of AAV. In this study, three CF pigs received aerosolized *piggyBac*/AAV-CFTRdR to the airways. Two weeks after delivery, we observed a significant increase in Cl^- current in trachea and bronchus of freshly excised tissues in response to the CFTR anion channel agonists forskolin and IBMX. We also observed a decrease in Cl^- current upon treatment with GlyH-101 in trachea and bronchus tissues, consistent with CFTR. Unlike the naïve control CF pigs, vector treated pigs exhibited an increase in tracheal ASL pH and bacterial killing to near wild-type levels. Trachea and bronchus cells were subsequently cultured and grown at an air liquid interface and assayed for a change in ASL pH and viscosity. Cultures from CF pigs treated with *piggyBac*/AAV-CFTRdR had an increase in ASL pH and decreased viscosity, consistent with improved CFTR channel activity. These exciting results set the stage for future evaluation of persistence and immune response preclinical studies.

554. New and Smaller MiniPromoters for rAAV-Based Expression in the Brain and Eye

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Small human promoters, capable of driving cell-type restricted gene expression, will be critical for the success of many gene therapies. Such promoters will restrict expression and thus limit off-target side effects and immunogenicity, show a more physiological-like expression than the often-used viral promoters, and contain exclusively human sequences that will be less susceptible to inactivation and more acceptable to regulatory agencies. However, the conversion of typically large human promoters into MiniPromoters for use in such space-limited vectors as recombinant adeno-associated virus (rAAV) presents a substantial challenge.

For this study, ~50% of candidate promoters were new bioinformatics-driven designs from genes with therapeutically interesting expression patterns, and ~50% were adapted from our previous published work. All promoters were cloned into a custom rAAV genome driving emerald GFP (EmGFP), and including a chimeric intron, woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), and SV40 polyA, and then packaged into rAAV9 at the University of Pennsylvania Vector Core. All viruses were tested by intravenous injection of 5e11 genome copies into newborn B6129F1 mice. A subset of MiniPromoters was further characterized by direct injection into the adult eye, including subretinal and intravitreal routes. Analysis included GFP immunofluorescent staining of expression driven by the MiniPromoter compared to the ubiquitous promoter smCBA. For a subset of promoters, co-staining with relevant markers and quantification of epifluorescent signal was also undertaken.

Successful promoters ranged in size from 682 to 2,982 bp. New promoters have been derived from *TUBB3* for ganglion cells of the retina, and *POU4F1* for corneal nerves. Smaller and more specific promoters were derived from *PCP2* - one for the Bipolar ON cells of the retina and a second for the Purkinje cells of the brain. Smaller promoters have now been derived from *CLDN5*, a gene expressed in the endothelial cells of the blood brain barrier; *FEV*, for the raphe of the brain; and *NR2E1*, for the Müller glia cells of the retina. Finally, four MiniPromoters previously tested with icre (improved cre, a very sensitive assay) are demonstrated to be strong enough to drive specific EmGFP expression: *NOV*, enriched in the cortex of the brain; *OLIG1*, for oligodendroglia of the brain; *PITX3*, for the stroma of the cornea; and *S100B*, for astrocytes in the brain.

All published Pleiades (Ple) MiniPromoters are available to the gene therapy community through Addgene (www.addgene.org), and unpublished materials are available by contacting the authors.

555. Brain Endothelial-Targeted Gene Therapy in a Mouse Model of Incontinentia Pigmenti

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Introduction: *Incontinentia pigmenti* (IP) is a rare genetic disease predominantly affecting the skin. The disease is caused by inactivating

mutations in the *Nemo* gene encoding the regulatory subunit of the inhibitor of kappaB kinase complex (IKK). One third of IP patients develop severe neurological symptoms caused by the death of brain endothelial cells and a disruption of the blood-brain barrier (BBB). As there is no conventional therapy for IP, AAV-mediated gene therapy may provide a beneficial treatment option.

Methods: An AAV2 mutant designated "AAV-BR1" with tropism for the brain endothelium was used to deliver the Nemo gene into IP mice. As opposed to humans, mice with heterozygous deletion of *Nemo* die of skin manifestation at early age. To circumvent this problem, mice with conditional knockout of the *Nemo* gene in brain endothelial cells were used as IP model. AAV-BR1 vector was delivered intravenously into 10-20 weeks old mice at a dose of 1.8×10^{11} vg (AAV-BR1-CAG-NEMO or AAV-BR1-CAG-GFP as control). Cortical screws for the recording of electroencephalograms (EEGs) were stereotactically implanted three weeks after vector injection. Tamoxifen treatment for induction of the conditional *Nemo* knockout was started three weeks after electrode implantation. Mice were continuously monitored for seizures by EEG and (infrared) video-recording within a two weeks period. BBB integrity was analyzed by quantification of albumin and IgG in brain extracts and endothelial cell death was assessed by quantifying the so-called string vessels as the remaining empty basement membranes of formerly intact microvessels.

Results: Delivering the *Nemo* gene into brain endothelial cells of IP mice by the brain-targeted AAV2 mutant AAV-BR1 prevented cell death and substantially improved the integrity of the BBB. String vessels were significantly reduced in the treatment group compared to the control group ($n = 11$, $p = 0.0002$). The time until the first electrographic and clinical seizures occurred during recording was significantly prolonged in the AAV-BR1-CAG-NEMO treated group compared to controls (all seizures: 8.7 ± 1.1 vs. 4.3 ± 0.4 days, $p = 0.0012$; clinical seizure: 8.8 ± 1.1 vs. 4.8 ± 0.5 days, $p = 0.0043$). Five out of 15 mice treated with AAV-BR1-CAG-NEMO did not suffer from any seizure during EEG recording, whereas all mice of the control group developed seizures (15/15). In addition, the number of mice exhibiting focal seizures was significantly reduced in the AAV-BR1-CAG-NEMO treated mice (8/15) compared to AAV-BR1-CAG-eGFP controls (14/15, $p = 0.0352$).

Conclusion: Our data show that AAV-mediated gene therapy may be a promising treatment option for diseases with severe neurological implications such as IP. Brain endothelial cells represent a promising therapeutic target potentially also for other neurovascular diseases. Interestingly, at least in IP, it does not seem to be necessary for the viral vector to cross the BBB to ameliorate neuronal symptoms such as epileptic seizures.

556. Highly Efficient Brain Neuron Targeting and Hepatocyte Detargeting with a Novel AAV9-PHP.B Capsid Harboring a Liver-Detargeting Mutation

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Global transduction of the central nervous system by systemic administration of AAV9 vectors is a compelling feature of AAV vector-mediated gene therapy. However, the tropic promiscuity of AAV9 proves to be a double-edged sword when developing AAV vectors for targeted clinical therapies. One key obstacle is the dissemination of AAV to the liver following intravenous administration. High levels of hepatic transduction result in: 1) decreased bioavailability to non-hepatic tissues, 2) increased risk of CTL-mediated liver damage, and 3) potentially increased risk of AAV-induced hepatocellular carcinoma. Here, we report that substitution of a single amino acid in the AAV9 capsid with alanine (AAV9LDmt) efficiently detargets the liver while maintaining levels of brain neuronal transduction comparable to wild-type (WT) AAV9. We also show that this liver-detargeting mutation can be applied successfully to the AAV-PHP.B capsid, the most robust mutant for brain transduction at present. In this study, we constructed a library of DNA/RNA-barcoded AAV9 double alanine (AA) scanning mutants that express each mutant capsid-specific RNA barcode under the control of the U6 snRNA pol III promoter, and administered the library at a dose of $2x10^{13}$ vector genomes (vg) per kg to three 8-week-old C57BL/6 male mice by tail vein injection. This library contained 122 AAV9 AA mutants and covered the N-terminal half (amino acids 4-355 of 736) of the AAV9 VP1 protein. Various tissues were harvested 11 days post-injection, and the tropism and transduction efficiency of each mutant was ascertained by Illumina sequencing of DNA and RNA barcodes recovered from the tissues. Analysis of this large dataset identified 36 liver-detargeting AAV9 mutants, defined as mutants with less than 10% of the RNA barcode reads achieved by WT AAV9. Although many of the liver-detargeting mutants also showed impaired transduction in other tissues, one mutant, AAV9LDmt, retained a level of brain transduction comparable to that of WT AAV9. We hypothesized that this liver detargeting mutation could be applied to other constructs and chose AAV9-PHP.B, an AAV9 capsid with a 7-mer insertion that is capable of more than 40 times greater transduction of the brain than WTAAV9, to address this hypothesis. We constructed the following four self-complementary AAV vectors: AAV9-hSynI-GFP, AAV9LDmt-hSynI-GFP, AAV9-PHP.B-hSynI-GFP, and AAV9LDmt-PHP.B-hSynI-GFP, and injected 8-week-old C57BL/6 male mice with each vector intravenously at a dose of $3x10^{11}$ vg/mouse. Eleven days post-injection, we quantified neuron transduction by immunofluorescence microscopy and levels of vector genome dissemination to the liver by qPCR. While AAV9-PHP.B transduced up to 76% of brain neurons, substantial vector genome dissemination to the liver (18% of the level of WT AAV9) was observed. In contrast, AAV9-LDmt-PHP.B was able to transduce up to 66% of brain neurons (more than 10 times that of WT AAV9) while limiting vector genome

dissemination to the liver to 0.03% of that of WT AAV9. In conclusion, we utilized DNA/RNA Barcode-Seq technology to identify a mutation that detargets the liver to increase the safety of AAV vectors in systemic administration. This mutation retains efficient transduction of brain neurons and can be implemented in a rational design approach of novel vectors that may be of interest for clinical translation.

557. Chem-AAV: Chemically Modified AAV for Gene Therapy

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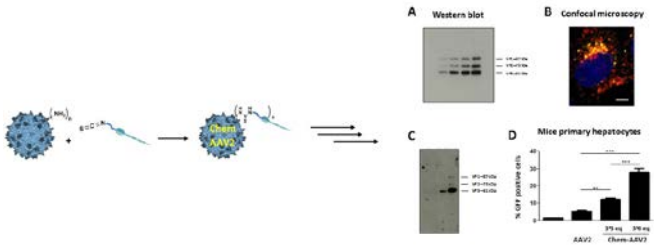
Recombinant AAV vectors are now becoming therapeutic products. However, several clinical trials using AAV have shown critical limitations: (i) high doses are usually required to achieve therapeutic effect; (ii) broad biodistribution to non-target tissues; (iii) loss of efficacy in the presence of pre-existing 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. As a proof of concept, a fluorescent probe was used to demonstrate the covalent coupling between an AAV2 and an organic molecule. For this purpose, the fluorophore molecule FITC, that contains an isothiocyanate chemical function (-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 FITC 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 FITC molecules grafted to the AAV particles according to the experimental conditions used in the chemical reaction (i.e. number of FITC Equivalents (Eq)/ AAV particles) (A). Using confocal microscopy it was possible to visualize FITC-AAV2 particles at the intracellular level upon transduction of HeLa cells (B). Interestingly, when AAV2 particles were labeled with high amounts of FITC (3e6 Eq/AAV particle), we noted an absence of recognition by the A20 antibody, suggesting that chemical modifications could decrease the sensitivity of Chem-AAV to preexisting neutralizing antibodies and/ or impair the humoral response to the modified capsid for *in vivo* studies. Based on these encouraging results, we decided to use our chemical modification technology to increase the specific targeting of AAV towards hepatocytes. To this end, *N*-acetylgalactosamine ligand (GalNAc) with a -NCS coupling function was synthesized since GalNAc recognizes the asialoglycoprotein receptor (ASPGR) present at the surface of hepatocytes. 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) (C). Importantly, GalNAc-AAV2 particles were more efficient in transducing mice primary hepatocytes than wild-type AAV2 and the efficacy was boosted by using increasing amounts of ligands (D). Altogether, our data shows the possibility to modulate at will the quantity and type of targeting ligands at the surface of the AAV particles and opens novel possibilities for gene transfer.



558. Comparative Next-Generation Sequencing (NGS) Analysis of a Single-Stranded rAAV DNA Packaged in Sf9 Insect, or HEK 293 Cells

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Recent advances in NGS technologies provide new tools to analyze the quality of GMP grade vectors and to make informed decisions on the process development and vector manufacturing platforms. Hereby, we describe the results of Illumina-based NGS analysis conducted for the purpose of comparing the degree of a genetic identity of encapsidated rAAV5 viral vector DNA manufactured by two alternative systems: 1) scalable, baculovirus infection-mediated insect cells, or 2) conventional HEK 293 cells co-transfected with bacterial plasmids. The insect cells platform consisted of Sf9-derived cell line incorporating AAV2 *rep* and AAV5 *cap* helper genes stably integrated into the genome. rAAV5 vector production was initiated by infection by a single recombinant baculovirus expression vector (BEV) harboring rAAV transgene cassette. The identical cassette was cloned into the bacterial plasmid to mediate rAAV production using standard co-transfection protocol in HEK 293 cells. The respective pseudotyped rAAV5s were extensively purified by sequential double iodixanol gradients, and their purified encapsidated ssDNAs were subjected to Illumina NGS side-by-side analysis. For bioinformatics, Fastq files were analyzed with the dedicated open source software ContaVect v0.2 modified to be used on a supercomputing cluster in its default configuration and were performed on the UF Research Computing HiPerGator supercomputer. Two main parameters were investigated: 1) the degree/origin of collateral packaging of contaminating DNA; and 2) the genetic identity of encapsidated transgene cassette. Direct side-by-side NGS analysis of the rAAV cassettes manufactured by two platforms revealed, unexpectedly, higher precision of viral DNA packaging in insect cells encapsidating considerably less contaminating DNA (0.6% vs 3.5%). Single nucleotide polymorphism (SNP) variants for DNAs from both viral samples, as well as positive control plasmid sample displayed very similar profiles of substitutions (correlation coefficient of 0.75-0.77). Interestingly, the majority of SNPs were co-localized with non-coding sequences identified as regions enriched in GC content, including AAV terminal repeats (TRs). Analysis of the genetic identity, therefore, showed no significant differences between the encapsidated rAAV DNA in insect vs human cells. However, we have established a previously undocumented fact of a genetic drift of

a plasmid DNA pool used to transfect HEK 293 cells. In summary, we provide an experimental evidence of significantly improved genetic quality of encapsidated rAAV DNA making insect cell a safer platform for GMP grade vector production.

559. Site-Directed Mutagenesis of an Engineered AAV3 Capsid Variant, LK-03, Improves Its Transduction Efficiency in Human Hepatic Cells *In Vitro* and in a Xenograft Murine Model *In Vivo*

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Of the 10 commonly used AAV serotype vectors, AAV3 was thought to transduce cells and tissues poorly. However, initial results from our laboratory indicated that AAV3 vectors transduce established human liver cancer cell lines as well as primary human hepatocytes extremely efficiently (*Mol. Genet. Metab*., 98: 289-299, 2009), since AAV3 utilizes human hepatocyte growth factor receptor as a cellular co-receptor for viral entry (*Hum. Gene Ther*., 21: 1741-1747, 2010). Subsequently, we documented significantly higher transduction efficiency of optimized AAV3 vectors, in which site-directed mutagenesis of specific surface-exposed tyrosine (Y), serine (S), threonine (T) and lysine (K) residues on the capsids was performed. The two best mutants (Y705+731F and S663V+T492V) were identified in human liver cancer cell lines *in vitro* and in a xenograft murine model *in vivo* (*Gene Ther*., 19: 375-384, 2012; *Hum. Gene Ther*., 25: 1023-1034, 2014). The enhanced transduction efficiency of the S663V+T492V mutant, compared with the wild-type (WT) AAV3 vectors, was also corroborated in the non-human primate (NHP) livers (*Mol. Ther*., 23: 1867-1876, 2015). Superior transduction efficiency of the WT AAV3 vectors was also reported by others in NHP livers (*Mol. Ther*., 23: 1877-1887, 2015), and of the optimized AAV3 vectors in human hepatocytes in humanized mice by us (*Mol. Ther*., 24: 1042-1049, 2016). Notably, Lisowski *et al*. described the isolation of a novel capsid variant, designated LK-03, through selection of a shuffled capsid library in a human liver xenograft mouse model (*Nature*, 506: 382-386, 2014). LK-03, which shares 97.7% homology at the DNA level, and 98.9% homology at the amino acids level with AAV3, demonstrated strong tropism in human hepatocytes *in vivo* in the humanized mouse liver. Since it still remains unclear as to which of the AAV serotypes (AAV3, AAV8, or LK-03) is the best vector for human studies (*Mol. Ther*., 23: 1800-1801, 2015), in the present study, we wished to evaluate whether site-directed mutagenesis of LK-03 could further augment its transduction efficiency. To this end, specific single (Y705F; S663V; T492V) and double (Y705+731F; S663V+T492V) mutations were introduced into the LK-03 capsid. No deleterious effect on the vector packaging efficiency was observed. However, in contrast to what was observed with the WT AAV3 vectors, whereas the two single-mutations (Y705F; S663V) had no effect, the two double-mutations (Y705+731F; S663V+T492V) significantly increased the transduction efficiency of LK-03 vectors by ~10-fold and ~6-fold, respectively. Next, the transduction efficiency of the best LK-03 mutant (Y705+731F-LK-03) was compared side-by-side with that of the best AAV3 mutant (S663V+T492V-AAV3) in 4 different human hepatic cell lines (Huh7, HepG2, LH86, HepRG) *in vitro*. No significant differences in the transduction efficiency were observed between these two optimized vectors in all 4 cell lines ($p>0.05$). Finally, the Y705+731F-LK-03 and the parental LK-03 vectors were injected via tail-vein into a human liver tumor xenograft murine model (n=4) *in vivo*. The efficiency of transgene expression in human liver tumors mediated by the Y705+731F-LK-03 vector was ~2.5-fold higher than that from its parental counterpart. Taken together, our results suggest that the strategy of site-directed mutagenesis of specific surface-exposed amino acid residues should also be applicable to other non-natural AAV variants for their optimal use in human gene therapy.

DZ and YZ: Equal contribution to this work; CL and AS: Co-corresponding authors

560. Gene Therapy to Rescue the Premature Stop Cystic Fibrosis Mutations R1162X and W1282X

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Premature stop mutations are represented within the 20 most common mutations in CF. Thus, to achieve the goal of treating everyone, therapies have to be developed restore function to patients with early stop codons. Several studies have shown that administration of the aminoglycoside, gentamicin, can partially rescue CFTR activity in CF subjects with Class 1 mutations. Although promising the rescue may not be large enough to provide clinical benefit. In this case patients with these mutations will need an alternative such as gene therapy. In previous studies we showed that transcomplementation of ΔF508 CFTR by Δ264 and Δ27-264 CFTR. Δ264 is missing the first 4 transmembrane segments of CFTR. Δ27-264 is similar to Δ264 CFTR but the first 27 amino acids of CFTR were added to the n-terminus. Both can rescue ΔF508 processing and chloride function by transcomplementation. We showed using FRET analysis and confocal microscopy that transcomplementation of ΔF508-CFTR occurs by a bimolecular interaction where the truncated form either Δ264 and Δ27-264 CFTR binds to ΔF508-CFTR within the ER and both travel to the plasma membrane where ΔF508-CFTR currents are restored. To address whether R1162X and W1282X function can be restored by transcomplementation we used CFBE41o$^-$ cell lines created in our laboratory stably using the Flip-in technology expressing each of these mutations. We infected the cells stably expressing either R1162X or W1282X with either AAV1 (2-7E+12 vg/ml) containing Δ264, or Δ27-264 or CFTRΔR-missing residues 708-759, known for its cAMP regulated channel activity. The purpose is to determine whether transcomplementation via AAV1 viral transduction could restore mutant CFTR function (assessed by short circuit current experiments) and compare it to the Cl^- transport induced by AAV1-CFTRΔR. Our results showed that transduction with Δ264 or Δ27-264 CFTR generated chloride currents that were approximately 2 fold greater than control for R1162X and 1.5 fold for than cells not infected with virus. The best effect was obtained with ΔRD transduction that generated 3.5 and 4.5 more current than control respectively for R1162X and W1282X, respectively. **Conclusion:** We provide evidence that R1162X and R1282X can be rescued to a small

extend with transcomplementation. However, the better option will most likely be achieved using AAV vectors that contain CFTR with generates chloride currents on their own.

561. NGS and Bioinformatics Pipeline for Targeted AAV Delivery by Directed Capsid Evolution

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The ability to target specific organs or tissues has been a recurrent goal of gene therapy, with the promises of decreasing the doses required to achieve therapeutic effect, limiting off-target unintended reactions, and minimizing or evading immune response. Directed evolution of AAV capsids using combinatorial libraries is a powerful, albeit challenging approach towards this goal. We describe here our strategy to monitor capsid diversity and variant enrichment during directed evolution, and how it informs decisions on which variants to select for evaluation or whether to continue the selection process. Although we have been using Illumina sequencing to characterize original libraries, the PacBio platform is more suitable during the directed evolution process, being able to produce long reads (our variants are in the order of 1-1.5 kb in size). We have developed a collection of Python scripts to address all bioinformatic needs, from raw data processing to detailed variant analysis. We show examples of applications, including the targeting of primate retinal tissue, human hepatocytes and human hematopoietic stem cells.

562. Abstract Withdrawn

563. PEO-PPO-PEO Micelles as Effective rAAV-Mediated Delivery Systems to Overexpress TGF-β in Human Osteoarthritic Articular Chondrocytes

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Introduction: Recombinant adeno-associated viral (rAAV) vectors constitute attractive tools for the durable treatment of human osteoarthritis (OA). Yet, their adapted use *in vivo* is impaired by some physiological barriers including the presence of neutralizing antibodies against viral capsid [1,2]. Delivery of rAAV via poly(ethylene oxide) (PEO) and poly(propylene oxide) (PEO-PPO-PEO) polymeric micelles is a novel, effective approach to overcome such hurdles, leading to a high and sustained expression of marker genes (E. coli *lacZ*) [3,4]. Here, we tested the feasibility of targeting human OA chondrocytes (hOACs) via rAAV using PEO-PPO-PEO polymeric micelles to overexpress the chondrogenic factor TGF-β. **METHODS:** rAAV-hTGF-β carrying a 1.2-kb hTGF-β cDNA controlled by the CMV-IE promoter/enhancer was packaged, purified, and titrated as previously described [5]. Poloxamer (Pluronic®) PF68 and Poloxamine (Tetronic®) 908 (BASF) were prepared in sucrose 10% at 4°C and mixed with rAAV (2% v/v final copolymer concentration) [3,4]. hOACs were isolated from unprocessed human OA cartilage, incubated with the rAAV/PF68 or rAAV/T908 mixtures, and maintained for up to 10 days at 37°C. rAAV in sucrose 10% (positive control) and sucrose 10% vehicle were used as positive and negative controls, respectively. TGF-β expression was monitored by TGF-β ELISA and immunohistochemical analysis. Cell proliferation was quantified using WST-1 and cytotoxicity via LDH. Proteogycan contents were monitored by alcian blue staining and spectrophotometrically estimated [4]. Each condition was performed in duplicate in two independent experiments and t-test and one-way ANOVA were applied to evaluate statistical differences between groups ($P \leq 0.05$). **RESULTS:** An evaluation of the TFG-β expression levels by ELISA revealed the effective rAAV-hTFG-β1-mediated gene transfer especially when the vector was provided via PF68 and T908 polymeric micelles compared with free vector treatment (up to 1.6-fold increase; $P \leq 0.04$). This tendency was observed by immunodetection of TGF-β (up to 1.6-fold increase; $P \leq 0.15$), exhibiting also a vector-dose dependent effect (up to 1.7-fold difference between 10 and 20 µl, $P \leq 0.01$). Overexpression of TGF-β increased hOACs proliferation, especially upon delivery of rAAV via polymeric micelles (up to 1.8-fold difference, $P \leq 0.002$ compared with the negative control). No cytotoxic events were noted in any condition (viability ≥ 90%; $P > 0.05$. Higher amounts of proteoglycans were always detected upon delivery of rAAV-hTGF-β via polymeric micelles (up to 1.5-fold difference, $P \leq 0.002$ compared with the negative control). **CONCLUSIONS**: Delivery of rAAV via PEO-PPO-PEO polymeric micelles allows for the safe, effective, and prolonged overexpression of the chondrogenic factor TGF-β in hOACs. Administration of therapeutic rAAV via PEO-PPO-PEO copolymers may be a powerful strategy to increase the residence time of the vectors at a target site for improved cartilage regenerative processes. **REFERENCES**: [1] Calcedo et al., Front Immunol 2013 [2] Rey-Rico & Cucchiarini, Acta Biomater 2016 [3] Rey-Rico et al., Acta Biomater 2015 [4] Rey-Rico et al., ACS Appl Mater Interfaces 2016 [5] Venkatesan et al., J Trans Med 2013. **ACKNOWLEDGMENTS**: Deutsche Forschungsgemeinschaft (DFG RE 328/2-1) and MINECO (SAF2014-52632-R and MAT2013-40971-R).

564. Simple Methods to Increase AAV Vector Production by Improving Transduction and Altering Downstream Processing

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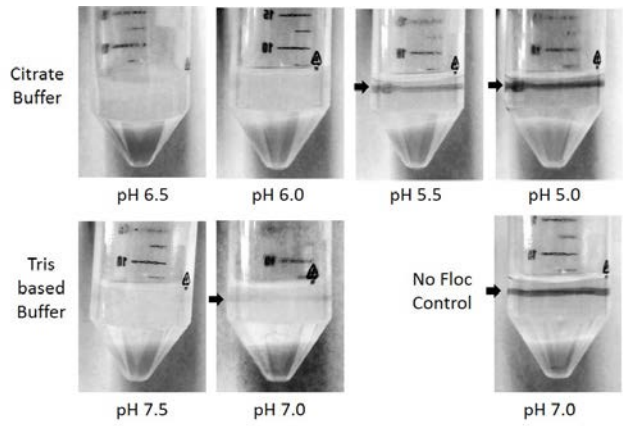
Adapting AAV vector production with new approaches is valuable. Over the past 20 years titers have dramatically increased by utilizing improved methods. Still, further modifications can be made to enhance the process. Our lab developed a novel approach to improve plasmid transduction and made variations to downstream processing to increase the titer. These new methods are simple to implement and do not require the addition of extra resources. Evidence shows (PMID: 20043217) that heat shocking cells at 42C improves endocytosis

and a recovery period is required to maximize uptake and prevent unwanted cellular death. Our lab tested if heat shocking improves calcium phosphate (CaHPO4) transduction by increasing uptake of the precipitated DNA. First we did small scale optimization using 6-well plates. From these initial trials we showed heat shocking cells at 42C for 1 hour and then allowing recovery for 2 hours at 37C increased transduction 2 to 3 fold using an eGFP expressing plasmid without cytotoxicity. Longer periods of heat shock and/or shorter periods of recovery caused cytotoxicity. During vector production, 1/2 of the cell factory was heat shocked while the other 1/2 was not. We tested 3 serotypes (AAV1, AAV2 & AAV8) using the pDP helper plasmid and determined transduction by red fluorescence. Heat shocking significantly improved transduction ~2 fold and increased the final viral titer 1.6 - 2.1 fold. These data were from 6 different cell factories (2 for each serotype). We also modified downstream processing. Evidence from Potter *et al*. (PMID: 26015974), shows lysing cells in citrate buffer and clearing the lysate under low pH conditions does not impact rAAV infectivity and improves flocculation. Floc following centrifugation is visible by a partially (but not completely) transparent supernatant. When using the standard tris-based resuspension or lysis buffer at more neutral pHs centrifugation speeds are typically 3000 - 4000gs at 20 to 30 mins. Even at these centrifugation speeds and times floc is still evident in the supernatant, which is typically not 100% transparent. It was our goal to reduce the centrifugation speed and time to clear the lysate based on improved flocculation using a low pH citrate buffer and ensure minimal or no floc was in the supernatant. We cleared the lysate at different pHs using citrate buffer. The centrifugation was done at 2000g for 10 mins. At pH 5.0 no floc was evident in the supernatant, which was completely transparent (a line was drawn on the back of the tube to assess transparency of the supernatant).



With qPCR we also showed that using more neutral tris-based buffers to clear the lysate at 3000g for 30 mins or 4000g for 20 mins recovers ~60% (ratio of 2:1 cleared lysate to pellet) of the rAAV vector from the lysate. Using the pH 5.0 citrate buffer at 2000g for 10 mins recovers ~80% (ratio of 4:1 cleared lysate to pellet) of the rAAV vector from the lysate. After clearing the lysate, iodixanol gradients and tangential flow filtration was used to purify and concentrate the viral vectors and facilitate buffer exchange. Titers were done using qPCR. Overall, when we combined the heat shock and recovery period of the cell factory with a low pH citrate buffer and slower and shorter centrifugation to clear lysate, we got an average total of 3.9e12 rAAV vector genomes (vgs) recovered from 1/2 of a cell factor which, if adjusted for a full cell factory, would be 7.8e12 vgs.

565. Triton Micellesinterference with AAV Vectors Adsorbing to AVB-Sepharose Columns

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Manufacturing of recombinant adeno-associated (rAAV) gene therapy vectors often involves lysing of the cell culture at harvest followed by affinity chromatography purification using AVB-Sepharose resin. Several mechanical and non-mechanical means can be used to lyse cells, however at a bioprocessing scale, detergent-based cell lysis, particularly Triton X-100, is both scalable and economical. Triton X-100, like other detergents, forms micelles of varying abundance and size, which are affected by concentration, temperature, and addition rate of Triton X-100 to the culture. Here, we demonstrate that the method used to add Triton X-100 to cell cultures can negatively impact the adsorbance of rAAV particles to AVB-Sepharose.

566. A Novel Scalable Production Platform for Viral Vectors Based on Human Suspension Cell Lines

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A rapid increase in the number of gene therapy trials and products has led to an equally increased need for industrial production of viral gene therapy vectors such as lentiviral, adeno-associated and adenoviral vectors. Current production systems are limited with respect to scalability and robustness. An adeno-associated virus (AAV) based vector was the first approved gene therapy product. 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. Production of high amounts of AAV meanwhile still faces challenges concerning upscaling and stability. The same holds true for production of lentiviral (LV) vectors, whose advantages are achievement of long-term expression of transgenes by integration into the genome and efficient transduction of dividing and non-dividing cells. One major area of application of LV based vectors is the CAR-T cell therapy field. With our CAP and CAP-T cell lines, we have developed a novel system for high density suspension culture, efficient reproducible transfection and high efficiency production of viral vectors. To optimize the production of viral vectors, we applied a design of experiments approach - the Box-Behnken design. Titration of supplements, cell density and DNA amount resulted in a significant increase of viral titers. In addition, viral production in shake flasks was shown to be scalable yielding comparable titers over a range of volumes. To address the need for scalable transient transfection without medium exchange step, we developed successfully a scalable protocol for transfection and viral production in spent medium.

567. Detection of AAV Vector DNA and Transgene RNA in Liver Tissue by Fluorescent In Situ Hybridization

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The liver is a pivotal organ in the metabolism and the center of numerous inherited metabolic disorders. Gene therapy represents a promising therapeutic approach for this class of diseases. Although high and long-term hepatic expression has been achieved in preclinical and clinical studies using AAV-based vectors, questions remain about the efficacy of liver cell transduction, such as the percentage of hepatocytes that are ultimately targeted and the spatial distribution pattern of transduced cells. This is of particular importance for diseases in which a high protein expression or high percentage of transduced hepatocytes is required to ameliorate the phenotype, such as metabolic diseases in which toxic metabolites accumulate due to insufficient enzymatic activity. However no standard laboratory techniques are currently available to answer to these questions.

To address this need, we applied the fluorescent in situ hybridization methodology to the specific detection of AAV vector DNA as well as transgene RNA in liver tissue. Specific fluorescent probes for AAV vector DNA and transgene mRNA were designed and used for liver tissue hybridization. Liver cell type specific antibody staining was performed in parallel. Images were acquired with confocal microscopy and analyzed with the use of an image analysis software (HALO, indicalabs).

To illustrate the potential of the approach, we performed an extensive assessment of the liver tissue transduction pattern in mice that were injected intravenously with AAV1-GFP or AAV1 hFIX. The analysis was based on the percentage and scoring of cells positive for AAV vector DNA and transgene RNA in relation with spatial distribution in the liver tissue and cell type determination. The results obtained allowed us to determine with a high sensitivity and accuracy the profile of AAV1 transduction and transgene expression in relation with tissue spreading of the AAV vector DNA and liver morphology.

In summary, we have shown that we can assess the physiological transduction profiles of AAV in the liver, a valuable tool for developing future liver gene therapies.

568. Widespread Transduction of the Central Nervous System Following Systemic Delivery of AAVHSC17 in Non-Human Primates

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Adeno-associated viruses derived from hematopoietic stem cells (AAVHSC) have been identified and cloned from normal human peripheral blood CD34+ cells. Sequence analysis of these novel AAVHSCs map to AAV Clade F, of which AAV9 is a representative member. Clade F AAVs are emerging as successful gene therapy vectors, particularly for transduction of the central nervous system (CNS) after systemic delivery. In the current study, the bio-distribution of a novel Clade F AAV, AAVHSC17, was compared to that of AAV9 in 3- to 4-month old male cynomolgus macaques (*Macaca fascicularis)*. Recombinant AAVHSC17 and AAV9, each packaging a self-complementary green fluorescent protein (scGFP) transgene driven by the chicken beta actin (CBA) promoter, were prepared by triple transfection in HEK293 cells and purified by double banding in cesium chloride density gradients. Animals were pre-screened for anti-AAVHSC17 and -AAV9 neutralizing antibodies (Nab). Nab negative animals (n = 2/group) received a single intravenous (IV) [1 x 10^{13} or 1 x 10^{14} vg/kg] or intrathecal (IT) [1 x 10^{13} vg/kg] injection of AAVHSC17 or AAV9. On Day 14 animals were euthanized and perfused with saline followed by 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Tissues were collected and fixed for 48h in 4% PFA in PBS. Bio-distribution of AAVHSC17 and AAV9 was assessed by GFP immunohistochemistry (IHC) on 40 µm frozen sections of the brain and spinal cord and on 4 µm sections of paraffin-embedded non-CNS tissues. IV administration of AAVHSC17 or AAV9 produced widespread distribution of GFP expression in astrocytes throughout the brain with the highest levels seen in the pons and in the lateral geniculate nuclei. GFP positive neurons were also observed throughout different regions of the brain. GFP expression was evident in axons of the lateral dorsal white matter tracts in the rostral spinal cord and axons and neurons in the dorsal and ventral gray matter of the rostral and caudal spinal cord. Compared to IV-treated animals, IT injections of AAVHSC17 produced less GFP expression in the brain but more pronounced staining of axons and neurons in the ventral horn of the caudal spinal cord. Widespread GFP expression in non-CNS tissues was observed in animals receiving IV AAVHSC17 or AAV9 with prominent staining in hepatocytes, skeletal- and cardio-myocytes. GFP expression in CNS and non-CNS tissues was dependent on dose of AAVHSC17 administered. These data demonstrate that AAVHSC17 has a broad tissue tropism and is able to effectively cross the blood brain barrier following systemic delivery in non-human primates, making it amenable for potential therapeutic applications in treating human genetic diseases.

Adenovirus Vectors and Other DNA Virus Vectors

569. Pathogenesis of Human Adenovirus Type 5 and 6 in Permissive Immunocompetent and Immunosuppressed Syrian Hamsters

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We and others have developed the Syrian golden hamster as a permissive immunocompetent model to evaluate the anti-tumor activity and toxicity of oncolytic adenovirus (Ad) vectors based on Species C Ads (Ad1, Ad2, Ad5, Ad6). In addition, we are studying

Syrian hamsters as a permissive model to understand Species C Ad pathogenesis based on the belief that some of our findings will apply to Ad pathogenesis in humans. Adenoviruses are generally benign in immunocompetent patients, causing infections of respiratory, GI, ocular, kidney, and other tissues. However, adenoviruses can be extremely dangerous in immunosuppressed patients, in which adenoviruses can cause disseminated disease and death. There is a great need to understand Ad pathogenesis in immunocompetent and immunosuppressed patients, and we believe that our models can provide useful information. We find that following intravenous (i.v.) administration of Ad5 or Ad6 into immunocompetent hamsters, the virus infects the liver and other organs, resulting in a robust immune response that eliminates the virus in about one week. In a STAT2 knockout Syrian hamster (with defective innate immunity), following i.v. administration of Ad5, replication levels were 100-1000-fold higher in the liver and other organs than in wild type hamsters, indicating that the type I interferon response is crucial to controlling Ad5 infection. The adaptive immune response remained intact in these STAT2 KO hamsters. Using a custom microarray platform, developed based on our complete sequence of the Syrian hamster transcriptome, we showed that type I interferon and other antiviral genes were robustly up-regulated in the liver at 18 h post infection of immunocompetent hamsters with Ad5. These microarray data are consistent with the STAT2 KO hamster data. When hamsters are severely immunosuppressed using high-dose cyclophosphamide, Ad5 and Ad6 replicate in the liver and other organs to very high levels and for extended time (weeks), similar to what can occur in immunosuppressed patients. In other studies, in work in accord with the Michael A. Barry lab, we found that Ad6 is much more pathogenic than Ad5 (the LD90 of Ad6 is 10-fold lower than that of Ad5), probably because Ad5 is sequestered more readily by Kuppfer cells than is Ad6. In other interesting work, we found that male hamsters are much more susceptible to Ad5 or Ad6 i.v. infection than are females. In still other studies with our immunosuppressed hamster model, we found that brincidofovir, cidofovir, ganciclovir, valganciclovir, and certain other anti-viral drugs are very effective against intravenously administered Ad5 or Ad6, suppressing virus replication and mitigating pathogenesis. Further, valganciclovir and cidofovir inhibit Ad6 replication and pathogenesis in the lungs of immunosuppressed hamsters after intranasal challenge. These data support the idea that these compounds could be used to treat immunosuppressed patients with disseminated Ad infection. These various findings should be of interest to investigators studying oncolytic Ad vectors for cancer therapy.

570. Overcoming Chemoresistance Through Degrading Extracellular Matrix and Inhibiting Metastasis of Pancreatic Cancer by Decorin and Wnt Decoy Receptor Co-Expressing Oncolytic Adenovirus

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Pancreatic cancer is a highly lethal disease and notoriously difficult to treat. Only a small proportion of pancreatic cancer patients are eligible for surgical resection whilst conventional chemoradiotherapy only has a modest effect with substantial toxicity. Gene therapy has become a new widely investigated therapeutic approach for pancreatic cancer. The extracellular matrix (ECM) proteins and epithelial to mesenchymal transition (EMT) phenotype regulate pancreatic cancer cell resistance to anticancer drugs. To enhance chemosensitivity of pancreatic cancer cells, we constructed cancer specific oncolytic adenovirus (Ad) expressing decorin (DCN) and soluble Wnt decoy receptor (sLRP6E1E2), HEmT-DCN/sLRP6. Here we have investigated cell killing effect of HEmT-DCN/sLRP6 and a marked increase in cytopathic effect was observed when compared to ONYX-015. Similarly *in vivo*, HEmT-DCN/sLRP6 induced greater antitumor effect in pancreatic orthotopic model. In addition, we have investigated antitumor effect in combination of HEmT-DCN/sLRP6 with gemcitabine. *In vivo* tissue staining demonstrated significantly higher apoptotic level, ECM degradation, and lower EMT markers expression in tumor tissues treated with HEmT-DCN/sLRP6 plus gemcitabine compared to the treatment with control groups. The results presented here show the advantages of DCN- and sLRP6E1E2-coexpressing cancer-specific oncolytic Ad in pancreatic cancer. The greatly enhanced antitumor efficacy of HEmT- DCN/sLRP6 makes it a promising therapeutic agent in the treatment of pancreatic cancer.

571. Age and Organ-Based Sex Disparity of AAV Mediated Gene Expression

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There are numerous publications documenting differences in AAV vector mediated gene expression levels between male and female recipients in several species. All observations of a sex-based difference report higher expression levels in transduced males. Some reports are based on statistically significant results but many are anecdotal observations, and in some studies, no gender differences were seen. It remains unclear whether AAV-related sex differences are restricted to specific AAV serotypes and specific target host organs. To add to this AAV gender-based data, we have used AAVrh.10hα1AT, a vector that has demonstrated male > female gender differences in gene expression, to examine vector transduction and transgene expression before and after sexual maturity in mice as well as in numerous organs of the adult mouse using human alpha-1 antitrypsin (A1AT) as the reporter transgene. No significant difference in serum A1AT levels were observed between male and female neonatal 3 wk mice following intravenous AAVrh.10A1AT administration ($p>0.8$) whereas adult male mice had significantly higher serum levels of A1AT (male > female) as early as 4 days post vector administration ($p<0.001$). This difference persisted to 90 days, the last time point evaluated ($p<0.001$). In contrast, following intravenous administration of a serotype 5 adenovirus vector (another episomal-based DNA vector), coding for human A1AT, no gender difference in adenovirus mediated A1AT transgene expression was observed in adult mice suggesting the gender difference may be AAV specific ($p>0.3$). Following intravenous administration of AAVrh.10α1AT, to adult male and female mice, the AAVrh.10 vector genomes and mRNA were quantified in numerous organs as was serum A1AT protein levels. Interestingly, at 90 days post-vector (10^{11} genome copies) intravenous administration, the vector copy number was significantly higher in the male lung ($p<0.03$)

and the female spleen ($p<0.03$), but no significant differences were observed in liver ($p>0.09$), heart ($p>0.4$), quadriceps muscle ($p>0.06$) or diaphragm ($p>0.5$). Assessment of α1AT mRNA levels showed significantly higher levels in the male liver at day 90 ($p<0.006$), which were detectable as early as day 4 ($p<0.02$). No sex difference of human α1AT mRNA levels, either at day 4 or day 90, were seen in any other organs studied [e.g. day 90: heart ($p>0.4$), lung ($p>0.5$), quadriceps muscle ($p>0.1$), diaphragm ($p>0.3$) and spleen ($p>0.2$)]. Significantly higher A1AT serum protein levels were observed in males at all time points ($p<0.001$). In the context that most of the intravenous administered vector targets the liver, sex-biased decreased levels of serum A1AT levels likely results from gender-related, sexual maturity related expression at the transcriptional level.

572. Decoy Wnt Receptor (sLRP6E1E2)-Expressing Adenovirus Induces Anti-Fibrotic Effect Via Inhibition of Wnt and TGF-β Signaling

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Background: Aberrant activation of the canonical Wingless type (Wnt) signaling pathway plays a key role in the development of hypertrophic scars and keloids. This aberrant activation of the canonical Wnt pathway and its potent profibrotic effects suggest that Wnt pathway can be a potential target for the development of novel anti-fibrotic agents. In this study, we evaluated the anti-fibrotic potential of a soluble Wnt decoy receptor (sLRP6E1E2)-expressing non-replicating adenovirus (Ad; dE1-k35/sLRP6E1E2) on human dermal fibroblasts (HDFs) and keloid spheroid. ***Method:*** Wnt family member 3a and its effector β-catenin expression in keloid tissues were analyzed by hematoxylin and eosin and immunohistochemical staining. HDFs were transduced with dE1-k35/sLRP6E1E2 or control Ad vector (dEl-k35/LacZ), and then luciferase reporter assay for β-catenin activity was performed. Furthermore, change in type-I and -III collagen mRNA levels in HDFs were measured by quantitative real time PCR. Wnt intracellular signaling and secreted transforming growth factor-β1 (TGF-β1) protein expression were examined by western blot and the enzyme-linked immunosorbent assay, respectively. The effect of sLRP6E1E2 on the nuclear localization of β-catenin and Smad 2/3 complex was evaluated by immunofluorescence staining. Lastly, the expression levels of major extracellular matrix components were investigated by immunohistochemistry in keloid spheroids after transduction with dEl-k35/LacZ or dE1-k35/sLRP6E1E2. ***Results:*** Higher Wnt3a and β-catenin expression was observed in the keloid region compared to the adjacent normal tissues. The activity of β-catenin and mRNA expression of type-I and -III collagen were significantly decreased following treatment with dE1-k35/sLRP6E1E2. The expression of LRP6, β-catenin, phosphorylated glycogen synthase kinase *3 beta*, Smad 2/3 complex, and TGF-β1 were decreased in Wnt3a- or TGF-β1-activated HDFs, following administration of dE1-k35/sLRP6E1E2. Moreover, dE1-k35/sLRP6E1E2 markedly inhibited nuclear translocation of both β-catenin and Smad 2/3 complex. The expression levels of type-I and -III collagen, fibronectin, and elastin were also significantly reduced in keloid spheroids after treatment with dE1-k35/sLRP6E1E2. ***Conclusion:*** These results indicate that Wnt decoy receptor-expressing Ad can degrade extracellular matrix in HDFs and primary keloid spheroids, and thus it could be highly beneficial for treatment of keloids.

573. Adenovirus Vector-Induced Hepatotoxicity During the Early Phase of Adenoviral Treatment Is Attributed to Inflammatory Cytokine-Induced Leaky Expression of Adenovirus Genes

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Hepatotoxicity is the major adverse event following *in vivo* application of an adenovirus (Ad) vector. Two peaks of serum alanine aminotransferase (ALT), an enzymatic biomarker of hepatotoxicity, are detected in mice following Ad vector administration. The first peak, which is detected about 2 days after administration, is thought to be mainly induced by Ad vector-induced innate immune responses, including the expression of inflammatory cytokines. The second peak, which is detected approximately 10 days after Ad vector administration, is mainly caused by adaptive immune responses against Ad proteins. We previously developed a novel Ad vector containing the targeted sequences of liver-specific miR-122a into the 3′-untranslated region of the E4 gene to suppress the leaky expression of Ad genes (Ad-E4-122aT, Shimizu K. *et al.*, ***Mol. Ther. Methods Clin. Dev***. 2014). When compared with a conventional Ad vector, Ad-E4-122aT treatment resulted in a significant attenuation of Ad vector-induced hepatotoxicity during the late phase because of efficient suppression of the leaky expression of the Ad genes in the liver. Furthermore, Ad vector-induced hepatotoxicity was also significantly lower during the early phase in the mice receiving Ad-E4-122aT than that in those receiving a conventional Ad vector, although Ad-E4-122aT and the conventional Ad vector induced comparable expression levels of inflammatory cytokines in the spleen. These data led us to hypothesize that Ad vector-mediated induction of inflammatory cytokine production alone does not cause hepatotoxicity during the early phase. In this study, we examined the mechanisms responsible for the low levels of hepatotoxicity during the early phase following Ad-E4-122aT administration. First, to examine the involvement of Ad vector-induced inflammatory cytokines in the hepatotoxicity during the early phase, Rag2/Il2rγc double-knockout (DKO) mice lacking T, B, and NK cells were administered a conventional Ad vector. The levels of serum ALT in Rag2/Il2rγc DKO mice were significantly lower than in wild-type mice 2 days following administration of a conventional Ad vector. mRNA levels of inflammatory cytokines, including IL-6 and IFN-γ, in the spleen were largely reduced in Rag2/Il2rγc DKO mice compared with those in wild-type mice. These results suggest that inflammatory cytokines are involved in Ad vector-induced hepatotoxicity during the early phase. In addition, leaky expression levels of the Ad genes in the liver of Rag2/Il2rγc DKO mice were lower than in wild-type mice. Next, primary mouse hepatocytes were transduced with a conventional

Ad vector and Ad-E4-122aT in the presence or absence of IL-6. The viability of hepatocytes following transduction with a conventional Ad vector was significantly lower in the presence of IL-6 than that in the absence of IL-6. Ad-E4-122aT did not reduce cell viability in the presence or absence of IL-6. In addition, leaky expression of the Ad genes in hepatocytes treated with a conventional Ad vector was significantly elevated by addition of IL-6. These results indicate that Ad vector-induced inflammatory cytokines induced leaky expression of the Ad genes in the liver, leading to hepatotoxicity during the early phase.

574. A Fiber and Hexon-Modified Infectivity-Selective Oncolytic Adenovirus Escapes Liver Sequestration After Systemic Administration in a Pancreatic Cancer Model

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Oncolytic adenovirus has high potential for systemic cancer therapy. However, its efficacy upon systemic application has been quite limited to date. Unlike loco-regional therapy, systemic application of cancer therapy mandates better tumor distribution and transduction. When adenovirus vectors are injected intravenously into mice, most of the virus goes to the liver and can in high dosage lead to liver toxicity. One of the reason for liver tropism is that hepatocytes express high levels of the primary adenovirus receptor (CAR), 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 of systemic therapy is limited. Therefore, the improvement of cancer selective transduction and vector distribution to avoid liver sequestration would overcome the obstacles for systemic delivery and enable efficient systemic treatment of cancer with oncolytic Ad (OAd).

To improve the tumor transduction, we have generated the pancreatic cancer-targeted OAd in previous studies. AdML-VTIN, an oncolytic adenovirus targets the mesothelin protein, which is overexpressed in pancreatic cancer. AdML-VTIN showed selective and powerful anti-tumor effect against Panc-1 tumors in both intratumoral injection (i.t.) and intravenous (i.v.) injection. Interestingly, when we assessed viral distribution after i.v. injection to the nude mice, the liver sequestration of AdML-VTIN was lower than AdML-5WT (Ad5 fiber) at 48 hrs after injection. By day seven, the viral copy number of AdML-VTIN in the tumor was more than three orders of magnitude higher than WT. When we compared the therapeutic effect of i.v. and i.t. injections, the tumor volume significant decreased in both groups. Although the viral particle in the tumor with i.v. injection was about half of that with i.t. injection at 24 hrs after injection, the anti-tumor effect of systemic injection group was similar to that of local injection group. These results suggest that systemic injection of the mesothelin-targeted OAd showed significantly lower liver sequestration and better tumor accumulation.

Next, to further reduce liver sequestration and increases antitumor efficacy, we developed an improved vector which is a double-modified vector with both hexon- and fiber-modification. The mesothelin-targeted OAd was additionally modified by substitution of Ad5 hexon-hypervariable region 7 (HVR7) with Ad26 hexon-HVR7. We assessed liver distribution of the double-modified vector after i.v. injection. When the virus copy number was compared after 48 hrs, double-modification resulted in significantly decreased liver sequestration. These data suggest that hexon-modification allowed to escape from the liver. The detailed mechanism of this finding is currently being investigated and focuses on the viral uptake by hepatocytes and Kupffer cells. We believe that the fiber and hexon double-modified vector might be a promising vector to reduce hepatotoxicity and enhance systemic antitumor effect for systemic treatment of pancreatic cancer.

575. Efficient Production of DNA- and RNA-Containing Papillomavirus Gene Transfer Vectors in Cell-Free In Vitro Reactions

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It is well known that papillomavirus capsids can transduce cells with nonviral DNA plasmids. These vectors have a defined tropism for a broad spectrum of tumors making them attractive candidates for cancer gene therapy. Papillomavirus vectors encapsidating gene expression plasmids of interest are currently produced in 293TT cells, which express T-antigen. This production method is not suitable for clinical application due the inclusion of small quantities of the oncogenic T-antigen DNA in the preparation. In addition, packaging of plasmids expressing toxin genes would be precluded, due to toxicity to the producing cells. Therefore, we developed a T-antigen independent cell-free papillomaviral vector production system that allows the incorporation of DNA and, surprisingly, also RNA into viral capsids. We defined the optimal reactions conditions for packaging of several conformations of purified DNA, and also RNA, into purified L1/L2 capsids of several different human and animal papillomavirus types. The relative efficiency of packaging and transduction of closed circular, relaxed circular and linear DNA, and linear RNA, varied among types but up to 10^{11} infectious units per ml were routinely generated, in some cases. Notably, efficient killing of cells by vectors transducing Pseudomonas exotoxin A mRNA was observed. The vectors produced by our method were sensitive to the same entry inhibitors and neutralizing antibodies as cell-derived pseudovirions and were found to be infectious in an in vivo mouse model. In summary, we have developed a chemically-defined cell-free method to produce papillomavirus vectors that functionally resemble cell-derived vectors. This defined production system should be compatible with Good Manufacturing Practices and therefore useful to produce papillomavirus vectors for cancer cytotoxic gene therapy and other clinical applications.

576. Molecular Determinants Limiting Productive Replication of Human Adenovirus in Mouse Cells

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Co-evolution of viruses and their hosts results in an equilibrium allowing for viruses to persist in their target hosts and spread through the susceptible populations. Highly prevalent viruses evolved efficient mechanisms to subvert host immune defenses and, therefore, are highly tailored to the particular species due to the inter-species variation and dissimilarity in molecular components constituting the host defense systems in different species. Human adenovirus serotype 5 (HAdv5) is highly prevalent in humans, however, early reports showed that direct infection of mouse cells with HAd5 did not result in progeny virus production. To better understand specific factors limiting productive HAdv5 infection in mouse cells, we analyzed efficiency of HAdv5 infection for several mouse cell lines followed by genome-wide next generation sequencing (NGS) approach to analyze quantitative and qualitative viral transcriptome deregulation in mouse cells from 6 to 36 hours post HAdv5 infection compared to human cells. Infectivity studies showed that mouse lung KLN cells and mouse meduloblastoma PZP cell line are highly infectable by HAdv5, while mouse lung MLg, breast mammary EMT6, skin epithelial JB6, and mouse hepatoma BNL cells are relatively resistant to HAdv5 infection, compared to human lung A549 cells. Comparative NGS analysis of viral gene expression in mouse MLg and human A549 cells at 6h, 9h, 12h, 15h, 18h, 24h, and 36h post infection demonstrated that although at 24h and 36h time point, the mRNA amounts for viral genes were comparable for mouse and human cells, at earlier time points there has been a significant delay in viral gene expression in mouse cells, compared to human cells. We found that in addition to the known decrease in E1B55K expression, in mouse MLg cells E1A13S, DBP, E4ORF3, pIVa2, and UXP were expressed at significantly lower amounts compared to their expression in human A549 cells. Furthermore, in MLg cells translation of HAdv5 proteins was non-detectible, and no de-novo virus production was observed. The addition of mouse adenovirus MAV1 only slightly improved translation of HAdv5 genes in mouse cells. In contrast, mouse KLN cells supported replication of HAdv5 DNA that resulted in production of infectious particles and formation of plaques *in vitro*. Therefore, our study provides evidence for the first time that the restriction of HAdv5 replication in mouse cells is not absolute, but cell-type specific. We further identified a set of key under-expressed target genes that limit productive replication of HAdv5 in mouse cells. Because qualitative unraveling of HAdv5 transcriptional program is similar between human and mouse cells with only a temporal delay, our study suggests that over-expression of these target genes may allow for a productive HAdv5 replication in mouse system. Our study improves our understanding of basic adenovirus biology and may help in developing new HAdv5-based oncolytic vectors capable of productive replication in immunocompetent mouse tumor models.

577. High-Throughput Screening of an Engineered Human Adenovirus Library on a Panel of Osteosarcoma Cell Lines

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So far ~70 types of human adenovirus have been identified, with virus type-dependent tissue tropisms. Previous research has highlighted a series of applications using different adenovirus types as promising tools to develop tailored, disease specific therapeutic approaches. Oncolytic virotherapy is one of the most promising novel treatment concepts for cancer and has been actively studied in pre- and clinical trials. Since the efficacy of virotherapy depends on efficient transduction of targeted tumors, initial screening of a broad range of viral agents to identify the most effective vehicle is essential. Meanwhile, most studies only focus on two or few vectors for vector characterization. Therefore, high-throughput screening (HTS) will enable faster and systematic evaluation. We generated a novel adenovirus (Ad) library consisting of 32 wildtype human Ads representing all known species and 20 recombinant adenoviruses (rAds) labeled with measurable markers (GFP/Luciferase reporter cassette). The reporter-labeled adenovirus library enables HTS of tumor disease-specific cell lines to identify better candidates for oncolytic virotherapy. Here we report HTS of our adenoviral library in tumor cells derived from osteosarcoma, the most frequent primary cancer of bone in young adults. Three different human osteosarcoma derived cell lines MG-63, Saos-2 and U-2 OS were studied. Since osteosarcoma is the most common malignant tumor in dogs, a canine osteosarcoma cell line D17 was also included. Transduction efficiency measured by luciferase expression was compared to commonly used adenoviral vector type 5 (Ad5). In Saos-2 and U-2 OS cells we could identify adenovirus types from species B (Ad3, Ad16 and Ad21) and species E (Ad4) with comparable transduction efficiency as Ad5; while in MG-63 cell line Ad21, Ad35 and Ad37 showed higher transduction efficiency than Ad5. Interestingly, Ad5 showed the highest transduction efficacy in canine osteosarcoma cell line D17, followed by Ad20 and Ad4. Moreover, the most efficient adenovirus types identified from the luciferase assays were further investigated in virus internalization assays based on real-time qPCR and in vitro oncolytic assays based on cell lysis, as well as virus-derived GFP expression levels and CPE occurrence. These results confirmed the findings from the luciferase assays. As a next step to achieve tumor-selective viral replication, we plan to further engineer the identified candidate adenovirus types by deletion of the pRB binding region in E1A or replacing endogenous viral regulatory sequences with a tumor-specific promoter, such as the midkine (Mdk) or the osteocalcin (OC) promoters. Taken together, the exploration of natural human adenovirus diversity will help us to identify a number of novel vectors based on alternative types with cancer-specific tropisms and low seroprevalence.

578. Coagulation Factor X Protects Ad5 Vectors from Neutralization by Complement in Human Serum

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The ability of Adenovirus (Ad) gene therapy vectors to reach target cells and deliver therapeutic transgenes can be significantly affected by proteins in the blood, including coagulation factors, natural IgM antibodies, Ad-specific IgG antibodies, and the classical complement system. In mouse models, we have found that the ability of Ad5 vectors to bind coagulation factor X (FX) shields these vectors from attack by natural IgM antibodies and the classical complement system. This FX-mediated shielding greatly improves survival of Ad5 vectors in mouse serum and is also necessary for efficient liver transduction after intravenous injection of mice with Ad5 vectors. We undertook the current study to examine whether human FX protects Ad5 vectors in human serum in a similar manner. Unlike mice, humans have variable levels of prior immunity against Ad5 due to childhood Ad infections. We acquired 30 complement-preserved individual human sera from a commercial supplier. These human serum samples were screened for low levels of Ad5-neutralizing IgG antibodies in order to better compare with previous studies using serum from naïve mice. Twelve of the 30 human sera were classified as non-neutralizing. By comparing the ability of human serum to neutralize a control Ad5 vector and a mutant non-FX-binding Ad5 vector, we found that FX was able to protect Ad5 from complement-mediated neutralization in all of the twelve human serum samples tested, similar to the role of FX in mouse serum. Our data suggest that FX binding uniformly protects Ad5 from neutralization by complement in human serum.

579. Cancer-Specific Functionality of Canine Promoters to Expand Expression-Targeted Gene Therapy in Canine Tumors

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Cancer gene therapy is an outstanding approach to treating cancer that aims to deliver therapeutic genes to kill cancer cells. However, the feasibility of the routine use of cancer gene therapy in clinics has been restricted as the therapeutic gene generates an off-target effect in normal cells resulting in normal cell toxicity. Thus, it is important to explore mechanisms to achieve targeted delivery of therapeutic genes to cancer cells with reduced or no toxicity to normal healthy cells. Several studies have shown that transcriptional targeting is a successful strategy to promote targeted expression of the therapeutic gene in various cancers such as lung, breast and prostate cancer by utilizing tumor- upregulated promoters to drive the expression of therapeutic genes in a tissue- or tumor-specific manner. Selection of appropriate intermediate animal models is a basic requirement for the successful cancer gene therapy. Some unique characteristics such as inter-individual and intratumoral heterogeneity and genomic sequence instability similar to humans validate the dog as an outstanding animal model of cancer and other complex human diseases. Although a number of studies have shown high levels of tumor-upregulated expression of several promoters, including human telomerase reverse transcriptase (hTERT), survivin, chemokine receptor 4 (CXCR4) and progression elevated gene 3 (PEG3) in a variety of human cancers and murine models, none of these promoters have been investigated for their activity in the canine model. Our goal is to identify and investigate the activity of these promoters in various canine tumors. To accomplish this goal, we measured the expression level of these promoters in canine tumor cells, tumor tissues and normal tissues by either measuring GFP reporter gene expression level driven (PEG3 promoter) or by employing RT-qPCR for endogenous promoters such as CXCR4, TERT, and Survivin. Results showed negligible expression differences between canine normal and tumor cells for the PEG3 and TERT promoters, although these promoters showed increased tumor-specificity in the human and mouse model in several previous studies. However, canine Survivin (cSurvivin) and canine CXCR4 (cCXCR4) showed markedly higher expression in a variety of canine tumor cells when compared with most normal cells and tissues. To further validate these findings, we cloned the sequences of these promoters in GFP reporter plasmids to evaluate their activity in canine tumors using a series of cellular transfections and infections experiments, followed by measuring of GFP expression through flow cytometry, normalized to CMV-GFP expression. Results were congruent with RT-qPCR data providing high levels of canine tumor expression for cSurvivn and cCXCR4. Low levels of expression were observed for cTERT, in agreement with the RT-qPCR data. These findings will help us to generate conditionally replicating adenoviruses as well as to provide a platform for further development of transcriptional targeting mediated gene therapy for canine cancer.

Cancer-Immunotherapy, Cancer Vaccines III

580. Cord Blood NK Cells Engineered to Express IL-15 and CD19-Targeted CAR Show Long-Term Persistence and Potent Anti-Tumor Activity

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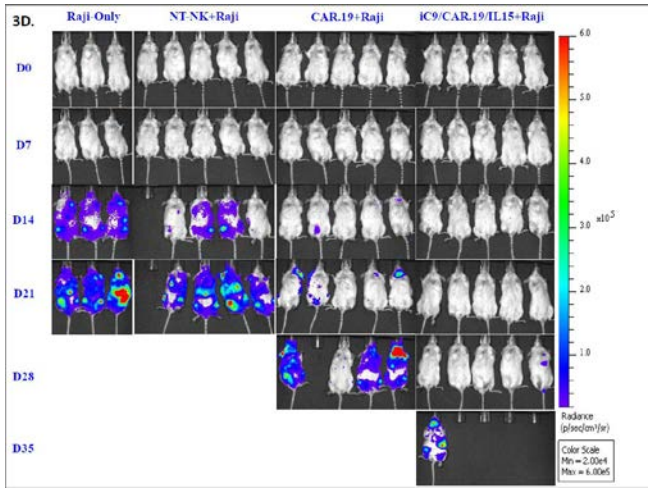
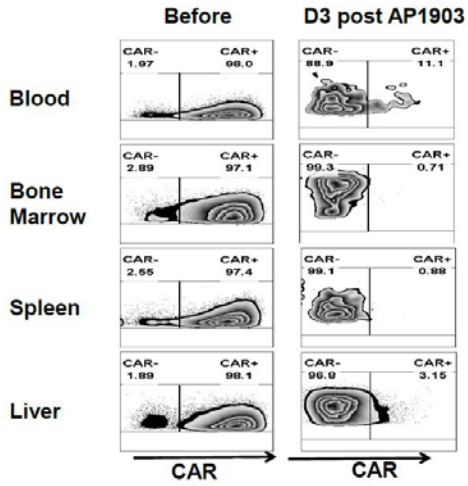
Chimeric antigen receptors (CARs) which redirect the specificity of autologous T-cells against leukemia and lymphoma are clinically very promising. To extend this approach to allogeneic T-cells is problematic

as they carry a significant risk of graft-versus-host disease (GVHD). Natural killer (NK) cells are highly cytotoxic effectors, killing their targets in a non-antigen specific manner without causing GVHD. Cord blood (CB) offers an attractive, allogeneic, off-the-self source of NK cells for immunotherapy. We transduced CB-derived NK cells with a retroviral vector incorporating the genes for CAR-CD19 (CAR.19), IL-15 and an inducible caspase-9-based suicide gene (iC9), and demonstrated efficient killing CD19-expressing cell lines and primary leukemia cells in vitro, with dramatic prolongation of survival in a xenograft Raji lymphoma murine model. IL-15 production by the transduced CB-NK cells critically improved their function. Moreover, iC9/CAR.19/IL-15 CB-NK cells were readily eliminated upon pharmacologic activation of the iC9 suicide gene. In conclusion, we have developed a novel approach to immunotherapy using engineered CB-derived NK cells which are easy to produce, exhibit striking efficacy and incorporate safety measures designed to limit toxicity. This approach should greatly improve the logistics of delivering this therapy to large numbers of patients, a major limitation to current CAR therapies.





581. Anti-CD123 CAR T-Cell Therapy for the Treatment of Myelodysplastic Syndrome

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Myelodysplastic syndrome (MDS) is a group of heterogeneous disorders caused by ineffective hematopoiesis that is characterized by bone marrow dysplasia and results in cytopenia. Currently, treatment options for MDS are limited to supportive care and hypomethylating agents, and most patients eventually succumb to the disease or progress to leukemia. Previously we have demonstrated that CD123 is an important target for the treatment of MDS due to its aberrantly high expression in high-risk MDS stem cells compared to non-stem cells and normal stem cells. In the present research, we generated a chimeric antigen receptor (CAR) vector containing a CD123-specific single-chain variable fragment in combination with a CD28 costimulatory domain, CD3ζ signaling domain and tEGFR. Anti-CD123 CAR was expressed on healthy donor and patient derived T lymphocytes utilizing lentiviral vector delivery to target high-risk MDS stem cells. Healthy donor derived anti-CD123 CAR T-cells effectively eliminated MDS cell line (>99% eradication) and primary bone marrow derived MDS stem cells (>70% eradication) *in vitro* (**Figure 1**). The killing was associated with increased cytokine release and CD107 degranulation by anti-CD123 CAR T-cells, which did not occur with control anti-CD19 CAR T-cells. Additionally, we successfully transduced T-cells obtained from patients with high-risk MDS to express anti-CD123 CAR with 50~70% transduction efficiency. Patient derived anti-CD123 CAR T-cells efficiently eradicated autologous CD123+ MDS stem cells (>99.9% eradication) *in vitro* compared to untransduced control T-cells (**Figure 2**). These results suggest that anti-CD123 CAR T-cells exhibit activity against high-risk MDS, and represent an effective therapeutic option for patients with high risk MDS. This work was funded by a grant from the Edward P Evans foundation.

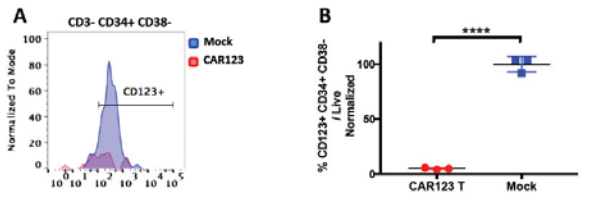

Figure 1. Healthy donor derived CAR T-cells eliminate primary high-risk MDS stem cells derived from patients' bone marrow in 48hrs. (A) Representative histogram and (B) percentage of CD123+ cells (normalized to mock) in primitive MDS stem cells (CD34+ CD38-) derived from patient bone marrow after 48hr co-cultured with healthy donor derived anti-CAR123 T-cells or untransduced T-cells.

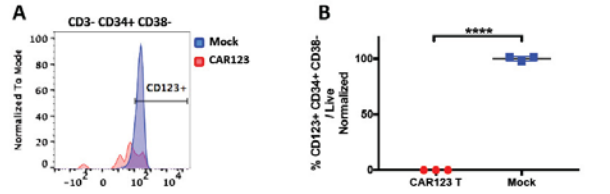

Figure 2. High-risk MDS patient derived CAR T-cells eliminate autologous MDS stem cells derived from their bone marrow in 48hrs. (A) Representative histogram and (B) percentage of CD123+ cells (normalized to mock) in primitive MDS stem cells (CD34+ CD38-) derived from patient bone marrow after 48hr co-cultured with anti-CAR123 T-cells or untransduced T-cells derived from the same patient.

582. Optimization of Gene Transfer Protocol Achieves Robust *In Vivo* Expression of DNA-Based Monoclonal Antibodies (DMAbs) in Small and Large Animals

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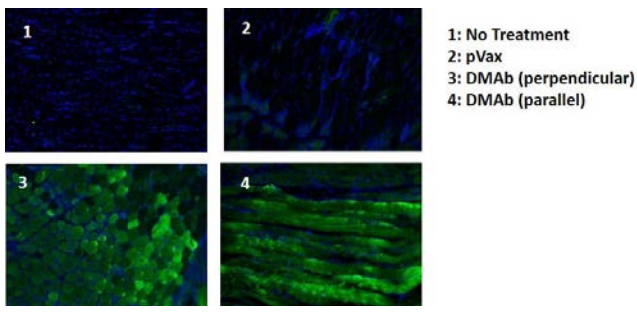
Monoclonal antibody (mAb) therapies have successfully been employed to treat various diseases. However, the high cost involved in the development of conventional protein mAbs has prohibited their global use. In response to this dilemma, gene therapy methods are being developed with the goal of delivering mAb encoding transgenes *in vivo*, and endowing the animal's own cells to function as antibody producing factories. One such technology is DNA-based monoclonal antibodies (DMAb). In proof of concept studies we have successfully employed this technology, where we deliver plasmid DNA (pDNA) vectors encoding monoclonal antibodies into the muscle cells of small animals to achieve systemic mAb levels. The mAb serum concentration is sufficient to protect against disease challenge. In this presentation we outline the development of a pDNA *in vivo* delivery platform which permits robust systemic antibody levels to be achieved in higher order species. Specifically, we have develop a platform based upon pDNA delivery with intramuscular (IM) electroporation combined with the ECM modifying FDA-approved drug, hyaluronidase. The image below shows representative Immunofluorescence images of in-vivo DMAb expression muscle tissue. Employing these methods we achieve robust serum levels (μg/ml) of functional human antibodies in mice, rabbits and non-human primates (NHPs). This optimized delivery platform supports the translation of DMAb technology into humans, and the potential for *in vivo* production of antibody levels capable of fighting a myriad of human diseases.



Immunofluorescence images of sections of the TA muscle treated with plasmid DNA encoding human IgG (DMAb) or pVax delivered with EP + HYA, and harvested 72 hours later. hIgG was detected with anti-human IgG followed by a FITC-labelled secondary antibody (green). DAPI stain in blue. Panel 1. No treatment. Panel 2. pVax. Panels 3 & 4. DMAb. Panels 1-3 display a cross-sectional image perpendicular to muscle fibers, and in Panel 4 the image is along the muscle fibers.

583. Biomimetic Artificial Antigen Presenting Cells Synergize with Anti-PD1 in the Treatment of Melanoma

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Introduction: Biomimetic artificial antigen presenting cells (aAPC) hold great potential for cancer immunotherapy. By coupling artificial Signal 1 and Signal 2 proteins to the surface of a polymeric particle, these aAPCs have shown to be capable of directing the activation of antigen specific CD8+ T-Cells.[1] However, despite this activation, the tumor cells are capable of suppressing the cytotoxic activity of these cells through various checkpoint proteins such as PD-1. Recently a new class of monoclonal antibody therapeutics has emerged to block the activation of PD-1 on T-Cells, yet efficacy of this biologic appears to be limited to a fraction of cancer patients.[2] In this study we investigated the combination of the aAPC and anti-PD1 for the treatment of melanoma.

Materials and Methods: Biodegradable aAPC were synthesized using Db IgG MHC dimer loaded with gp100 peptide to serve as Signal 1 and an antibody for CD28 to serve as Signal 2 as previously described.[1] To evaluate synergy between the aAPC and the anti-PD1 *in vivo*, we utilized an adoptive immunotherapy melanoma model. Four days prior to treatment, B6 mice were inoculated subcutaneously with B16/F10 melanoma cells. On the day of treatment, transgenic pMEL T-Cells were administered retroorbitally either by themselves, with the aAPC, with anti-PD1, or with a combination of the aAPC and anti-PD1. The animals were sacrificed upon a measured tumor area of 200 mm^2. In a separate experiment, the animals were all sacrificed eleven days post treatment and the tumor infiltrating lymphocytes (TILs) were evaluated for PD-1 expression.

Results and Discussion: The aAPC demonstrated a synergistic effect with anti-PD1. *In vivo* there was a noted 2-3 fold proliferative advantage in the antigen specific T-Cells in the combination aAPC/anti-PD1 therapy compared to either therapy alone. In addition, there was a significant reduction in tumor burden in the dual treatment group compared to the single treatment groups or the negative control. There was also a significant increase in survival in the dual treatment group compared to the single treatment groups (**Fig 1a**). The median survival time for single treatment groups was 17 days and the dual treatment group was 20 days. Isolated TILs in groups that received anti-PD1 demonstrated a 3-4 fold reduction in PD-1 surface expression compared to groups that did not receive anti-PD1 (**Fig 1b**).

Conclusions: In this study we have demonstrated a synergy between the use of aAPC for antigen specific T-Cell stimulation and an anti-PD1 checkpoint blockade. The combination of these two therapies resulted in an increased survival benefit compared to the single treatment groups. Continued investigation into dual treatment strategies such as this one could provide significantly increased efficacy for existing cancer immunotherapies.

References: [1]Sunshine, J., et. al. *Biomat*. 2016, [2]Callahan, M., et. al. *J. Leukoc. Biol.* 2013.

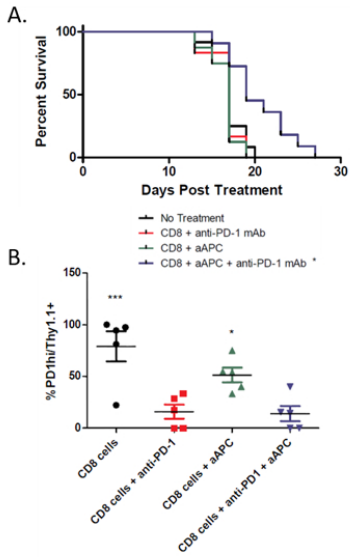

Figure 1. (A) Survival of mice in murine tumor treatment model is enhanced in dual therapy group. (B) TILs have decreased PD-1 expression in groups that received anti-PD1. (*=p<0.05, ***=p<0.001 compared to no treatment).

584. Adenoviral IL24 (Ad-IL24) Tumor Suppressor Immune Gene Therapy Reverses Checkpoint Inhibitor Resistance and Induces Abscopal Therapeutic Efficacy

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Immune checkpoint inhibitors represent an important advance in cancer therapeutics. However, the majority of cancer patients do not respond or become resistant to this form of immune therapy. Interleukin 24 (IL-24) is a tumor suppressor cytokine in the IL-10 family with multiple anti-cancer mechanisms including induction of apoptosis, inhibition of angiogenesis and activation of immune modulatory genes. We evaluated the ability of Ad-IL24 to reverse immune checkpoint inhibitor resistance and induce abscopal therapeutic effects in the highly immune therapy resistant murine B16F10 melanoma tumor model. To mimic clinical conditions of immune checkpoint inhibitor resistance, animals with established tumors were treated with anti-PD-1 before initiating Ad-IL24 intra-tumoral therapy. Consistent with previously published studies, anti-PD-1 had minimal to no therapeutic efficacy compared to control treatment. There was severe tumor size progression in animals treated with anti-PD-1 monotherapy and a modest decrease for primary tumors treated with Ad-IL24 alone. In contrast, there was a statistically significant reversal of anti-PD-1 resistance in tumors treated with combination Ad-IL24 + anti-PD-1 therapy. A statistical analysis of variance (ANOVA) comparison of tumor volumes for each treatment group revealed that the combined effect of Ad-IL24 and anti-PD-1 treatment was synergistic compared to either therapy alone (p-value = 0.002). Surprisingly, there was a statistically significant abscopal effect with decreased growth of contralateral tumors that were not injected with Ad-IL24 tumor suppressor therapy. A statistically significant decrease in abscopal tumor growth was observed in animals whose primary tumors were treated with Ad-IL24 + anti-PD-1 ($P<0.0001$) compared to the growth rate of tumors treated with anti-PD-1 alone. These findings imply that the combination Ad-IL24 + anti-PD-1 treatment induced systemic immunity mediating the abscopal effects. With respect to survival, combined Ad-IL24 and anti-PD-1 therapy demonstrated a statistically significant increase in survival compared to Ad-IL24 therapy alone ($p = 0.0167$) and anti-PD-1 therapy alone ($p < 0.001$) by the log rank test. Consistent with the synergistic effects on tumor growth, the increase in median survival for the combined Ad-IL24 and anti-PD-1 group was more than additive compared to the effects of Ad-IL24 and anti-PD-1 treatments. Overall, these results indicate that Ad-IL24 tumor suppressor immune gene therapy can reverse immune checkpoint inhibitor resistance and induce abscopal effects with statistically significant synergistic therapeutic efficacy. These findings support the planned clinical evaluation of combined Ad-IL24 and anti-PD-1 therapy in patients resistant to immune checkpoint inhibitor therapy.

585. Vascular Targeting Viral Therapy Augments Pd-L1 Checkpoint Blockade Anti-Tumor Activity

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Introduction: Immunotherapy with checkpoint inhibitors demonstrates remarkable efficacy in several cancers; yet, only a small percentage of patients profit from this class of treatment when given as a monotherapy. Several studies have shown that response rates are increased when checkpoint blockade is combined with other anti-cancer treatments. VB-111(Ofranergene obadenovec), is a biologic agent based on a non-replicating Adenovirus 5, that is currently in rGBM phase III clinical trial. VB-111 specifically targets angiogenic endothelial cells and promotes antitumor immune response. In this study, VB-111 was assessed for augmenting the effect of PD-L1 checkpoint inhibitor in lung and melanoma cancer models. *Experimental procedure:* In the Lewis lung carcinoma (LLC) model, 12-14 weeks old C57Bl6 mice were injected with $5x10^5$ D122 tumor cells. When the tumor diameter reached 7mm, it was removed and five days later, mice were randomly divided into different groups and treated with: i) saline administered systemically via tail vein injection; ii) VB-111 at $1x10^9$ viral particles (VP)/mouse administered systemically via tail vein injection; iii) 200µg/mouse anti PD-L1 monoclonal antibody administered intraperitoneally on days 5, 8 and 11; iv) combination of VB-111 at $1x10^9$ VP/mouse administered systemically via tail vein injection and 200µg/mouse anti PD-L1 monoclonal antibody administered intraperitoneally on days 5, 8 and 11. Upon sacrifice, lungs were harvested and weighted. Tumor burden of each animal was calculated by reducing the lung weight of age-matched normal mice. Lungs were also stained by immunohistochemistry with anti CD8 antibody for the presence of infiltrating cytotoxic T-cells. In the melanoma model, C57Bl6 mice were injected with $2x10^5$ B16F10

melanoma cells subcutaneously. When tumor size reached 100mm^3 (day 9), mice were assigned to different groups based on tumor volume and body weight and treated with: i) saline administered systemically via tail vein injection once on day 9; ii) VB-111 at 1x10^{11} VP/mouse administered systemically via tail vein injection once on day 9; iii) Anti PD-L1 200μg/mouse administered intraperitoneally on days 9, 12 and 14; iv) combination of VB-111 at 1x10^{11} VP/mouse administered systemically via tail vein injection on day 9 and 200μg/mouse anti PD-L1 monoclonal antibody administered intraperitoneally on days 9, 12 and 14. Tumor volume was measured throughout the experiment to determine efficacy. *Results:* In mice induced with LLC tumor and treated with saline, large tumor masses were observed in the lungs, and the calculated average tumor burden was 0.883g. Administration of the checkpoint inhibitor anti PD-L1 resulted in a reduction of 51% in lung tumor burden, compared with 42% in the VB-111 treated group. When VB-was combined with anti PD-L1, lung tumor burden was reduced by 67%. The number of infiltrating CD8 T-cells in mice receiving VB-111 or anti PD-L1 monotherapy was increased compared with saline treated mice. In the melanoma model, mice administered with anti PD-L1 displayed a notable reduction of up to 32% in tumor volume. Monotherapy with 1x10^{11} VB-111 showed increased effect over anti PD-L1 treated mice, reducing tumor volume up to 43%. However, combined treatment of VB-111 with anti PD-L1 profoundly augmented the effect of each monotherapy and reduced tumor volume by up to 57%. *Conclusions:* Our data demonstrate that treatment with VB-111 augments the anti-tumor activity of anti PD-L1 checkpoint inhibitor in lung cancer and melanoma pre-clinical models. These results support evaluating the efficacy of VB-111 and checkpoint inhibitors combination therapy in cancer patients.

586. Precise Glycoediting by CRISPR/Cas9-Mediated Gene Disruption Elucidates the Specificity of a Chimeric Antigen Receptor for the Globoside SSEA-4

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Stage-specific embryonic antigen-4 (SSEA-4), a globoside and characteristic marker of embryonic stem cells, was recently identified as a therapeutic marker for a broad array of cancer histotypes. In addition to glioblastoma, SSEA4 identified 65% of lung cancer lines, 71% of colon cancer lines, 74% of breast cancer lines, 89% of ovarian cancer lines, 100% of prostate cancer lines, and 100% pancreatic cancer lines. Here, we determined the sequence of the widely used and well-characterized anti-SSEA4 antibody, MC813-70, and developed a chimeric antigen receptor (CAR) using the scFv. We confirmed the expression of SSEA-4 in several tumor cell lines and demonstrated in vitro cytolytic activity by T cells genetically modified to expressed anti-SSEA4 CAR. While treating a xenograft model with CAR-T cells, we observed death of treated animals by lymphocytic and neutrophilic pleuritis and interstitial pneumonia and identified SSEA-4 as a marker of murine bronchial epithelial airways. Chromium release assays of normal human primary cells also demonstrated cytotoxicity by SSEA4 CAR-T cells, which poses a safety concern for further development of SSEA-4 CAR-T cells into a clinical product. Lastly, used CRISPR/Cas9-mediated gene-editing of multiple glycotransferases in the MCF-7 tumor cell line to precisely dissect and confirm the specificity of MC813-70 mAb and CAR-T cells for SSEA-4, a useful technique for characterizing antibody-based therapeutics that target glycosylated and non-protein targets. These data demonstrate that SSEA-4, a potential therapeutic target for a broad array of cancer histotypes, exhibits expression in normal lung tissue and thus obstructs clinical development of cellular-based therapy against the antigen. This is relevant to the development of glycolipid-targeting CARs and T cells targeting tumor antigens that are also expressed in normal human tissues.

587. ^{19}F-Labeled Human Peripheral Blood Mononuclear Cells Detected by Clinical MRI in a Therapeutic Cell Setting

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Background and Goal: Once injected, the fate of injected therapeutic cells *in vivo* becomes an unknown. For antigen presenting cell (APC)-based cancer vaccines, knowledge and quantification of their *in vivo* location is important as the number of cells reaching a secondary lymphoid organ is directly proportional to the magnitude of the ensuing immune response. We hypothesize that ^{19}Fluorine (^{19}F) cellular magnetic resonance imaging (MRI) as a non-invasive imaging platform can track and quantify *in vivo* therapeutic cell migration of peripheral blood mononuclear cells (PBMC), which contain APC. The goal of this study is to assess the feasibility of labeling human PBMC under Good Manufacturing Practice (GMP) conditions with a ^{19}F-perfluorocarbon (PFC) labeling agent and detect these therapeutic cells using a clinical MRI scanner.

Methods: Human PBMC were obtained from 5 subjects and labeled with ^{19}F-PFC (5mg/mL) overnight under GMP conditions. Viability (7-AAD) and lineage phenotype frequency was analyzed by flow cytometry. Nuclear magnetic resonance spectroscopy quantified ^{19}F uptake/cell. Pre-clinical nu/nu mouse 9.4T MRI studies were conducted following subcutaneous flank ^{19}F-PBMC injection. Clinical 3T MRI studies were performed with a dual-tuned ^{1}H/^{19}F resonance frequency (RF) surface coil following ^{19}F-PBMC injection into a ham shank phantom. A balance steady state free precession sequence acquired the ^{1}H/^{19}F images.

Results and Conclusions: We show near 100% PBMC labeling with ^{19}F-PFC without affecting viability or phenotype while maintaining sterility and quality assurance requirements. Following ^{19}F-PBMC injection into mice, injection site and popliteal lymph node detection of ^{19}F-PBMC was measured and quantified (Fig. 1); indicating that the manufacturing process is appropriate for an upcoming clinical trial. To optimize ^{19}F clinical MRI parameters, mock ^{19}F-PBMC injections into a ham shank permitted the minimum detection of 4.11x10^6 ^{19}F-PBMC

intradermally as well as 3.76x10^6 ^{19}F-PBMC at a depth of 1.2 cm (Fig. 1). This is the highest reported sensitivity of ^{19}F detection in the literature using a clinical MR scanner and surface RF coil.

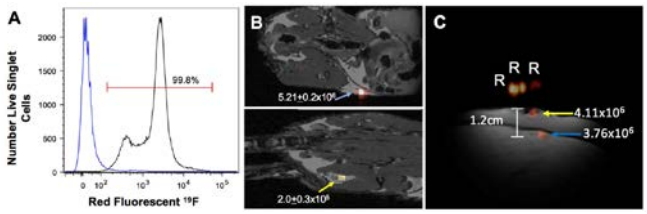

Fig. 1. Human PBMC labeled with ^{19}F-PFC under GMP conditions are detected by cellular MRI. 100% of PBMC were labeled with a fluorescent version of ^{19}F-PFC (5mg/mL) (black histogram, unlabeled PBMC blue histogram) (A). Pre-clinical *in vivo* mouse MRI detected ^{19}F-PBMC at the injection site (blue arrow, top panel) (B) and in the popliteal lymph node (yellow arrow, bottom panel) approximately 1.5 hours post injection. Clinical 3T MRI detected mock injections of 4.5x10^6 intradermal (yellow arrow) and subcutaneous (blue arrow) ^{19}F-PFC PBMC (C).

588. Optimized Sustained Local Co-Delivery of Dendritic Cells and Oncolytic Adenovirus Co-Expressing IL-12 and GM-CSF Using a Biodegradable Polymeric Reservoir for Cancer Immunotherapy Co-Delivery of Dendritic Cells and Oncolytic Adenovirus Co-Expressing IL-12 and GM-CSF Using a Biodegradable Polymeric Reservoir for Cancer Immunotherapy

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Administration of dendritic cells (DCs) combined with oncolytic adenovirus (Ad) expressing antitumor cytokines induces a potent antitumor effect and antitumor immunity by ameliorating the immunosuppressive tumor microenvironment. However, this combination therapy has significant limitations due to rapid dissemination and inactivation of the therapeutics at the tumor site, necessitating multiple injections of both therapeutics. In this study, we investigated injectable and biodegradable gelatin-based hydrogel as a carrier for oncolytic Ad co-expressing interleukin (IL)-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (oAd) and DCs to elicit a potent antitumor effect with single administration. Gel matrix enabled sustained release of both oAd and DCs while preserving their biological activity over a considerable time period, leading to efficient retention of both therapeutics in tumor tissue. Further, tumors treated with oAd- and DC-loaded gel (oAd+DC/gel) showed a significantly greater expression level of IL-12, GM-CSF, and interferon-γ (IFN-γ) than either single treatment (oAd or DC) or oAd in combination with DC (oAd+DC), resulting in efficient activation of both endogenous and exogenous DCs, migration of DCs to draining lymph nodes, and tumor infiltration of CD4$^+$ and CD8$^+$ T cells. Moreover, oAd+DC/gel resulted in a significantly higher number of tumor-specific IFN-γ-secreting immune cells compared with oAd+DC. Lastly, oAd+DC/gel significantly attenuated tumor-mediated thymic atrophy, which is associated with immunosuppression in the tumor microenvironment, compared with oAd+DC. Taken together, these results demonstrate that gelatin gel-mediated co-delivery of oncolytic Ad and DCs might be a promising strategy to efficiently retain both therapeutics in tumor tissue and induce a potent antitumor immune response for an extended time period via a single administration.

589. FnCARs: A Platform Expanding the Repertoire of Proteins Targetable with Chimeric Antigen Receptors

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CAR-T and -NK cells are promising tools for specific elimination of cancer cells. In most CAR designs implemented to date, recognition of target cells is mediated by scFv-based modules. This format, however, has a number of limitations, prompting identification of alternative antigen-recognition domains. One such variant is represented by an Fn3 scaffold (monobody), which offers the advantage of reduced immunogenicity, smaller size and higher stability compared to scFvs. Here, we for the first time describe the construction of a functional CAR with an Fn3 module replacing the conventional scFv. We introduced a VEGFR2-specific Fn3 domain to obtain a series of FnCARs with lightweight extracellular region. These VEGFR2-specific FnCARs display robust and specific activation properties and efficiently redirect cytotoxic activity of YT cells toward VEGFR2-positive target cells. Our proof-of-concept study shows that FnCARs are functional in vitro and suggest that Fn3 domains could potentially expand the repertoire of targetable cancer epitopes as well as streamline the design of bi- and poly-specific CARs.

590. Engineering the Skin to Enable a Pro-Immunogenic Cutaneous Microenvironment for Potent and Effective Vaccination

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The skin is a readily accessible organ that harbors keratinocytes and immunocytes, rendering it an ideal anatomical target for immunization. Keratinocytes initiate skin inflammation by producing pro-inflammatory mediators to regulate cutaneous immune responses. As such, targeting keratinocytes to increase production of antigens and pro-inflammatory mediators is salient and attractive for vaccination, but remains a critical challenge to achieve. Here, we demonstrate that transient overexpression of transcription factor x-box binding protein 1 (XBP1) in the skin not only enhances the expression of pro-inflammatory mediators and co-delivered antigen, but also increases skin-infiltrating immunocytes, including antigen-specific CD8$^+$ T cells and skin-resident memory CD8$^+$ T cells, that correlate with the

induction of protective antitumor immunity. Our findings show that keratinocytes genetically engineered *in vitro* with XBP1 and secreted antigen increased production of not only the exogenous antigen, but also pro-inflammatory mediators. Furthermore, transient *in situ* overexpression of XBP1 in keratinocytes sufficiently enabled a pro-immunogenic cutaneous microenvironment and enhanced vaccine-induced durable antigen-specific $CD8^+$ T cell and protective antitumor responses. Importantly, similar findings were observed in human skin: cutaneous transient overexpression of XBP1 triggered the expression of genes linked to a pro-immunogenic microenvironment and increased the expression of the co-delivered antigen. Our findings provide novel insights into the rational design of vaccines with greater immunization potency by way of strategic modulation of the skin microenvironment.

591. Genome Editing of Human T Cells to Generate CAR-T Cells with Enhanced Antitumor Function

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Toolgen, Seoul, Korea, Republic of

Innovative genome engineering technology utilizing programmable nucleases enabled a fast and efficient editing of genetic information in various cells and organisms. CRISPR/Cas9 nucleases, recently developed from a prokaryotic adaptive immune system, provide a robust programmable nuclease platform with high reliability and specificity. Here, we present the use of CRISPR/Cas9 nucleases for highly specific and efficient genome editing in human primary T-cell, without causing severe cell damage and growth retardation. Since it has been proposed that T-cell anergy serves to induce dysfunction in a wide variety of tumor microenvironment, we used CRISPR/Cas9-mediated gene editing to tackle the issue. First, we have successfully disrupted tolerance-associated gene, which allowed T-cells to reverse hypofunction induced by anergy. Based on the result, we combined the lentiviral delivery of CAR and electroporation of RNP to generate CRISPR gene-edited CAR-T cell. Compared to unmodified CAR-T, gene-edited CAR-T cells were less sensitive to immunosuppressive conditions, and hyperresponsive against tumor cells. The finding suggests that CAR and TCR T cells with disrupted anergy-related gene may be potent effector cells against various cancers.

Cancer-Oncolytic Viruses II

592. Human Oncolytic Adenovirus Bio-Distribution and Toxicity Differ Between Pigs and Mice

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The field of oncolytic adenovirus (OAd) therapy has undergone much growth in recent years as improvements in vector design have resulted in augmented selectivity and more potent anti-cancer effects. Before planning a clinical trial, the newly developed OAd must be tested in pre-clinical animal models to access efficacy and toxicity. However, the currently existing animal models provide very limited information regarding the vector performance in humans. Mouse models are insufficient because human Ad does not replicate in murine tissues, thereby barring the possibility to analyze OAd biodistribution and toxicity. Non-human apes have problems of availability and cost. Cotton rats and Syrian hamsters are able to permit OAd replication, but are not suitable for certain tropism-modified vectors. For example, Ad3-, Ad35-retargeted vectors cannot be tested since rodent cells lack CD46 and desmoglein, primary receptors recently identified for the B-group Ads. In this study, we explored a pig as a model to study performance of OAds, including those with Ad3-retargeted tropism. First, we demonstrated the ability of swine cell lines to support replication of Ad5 and Ad5/Ad3. Secondly, we analyzed binding, gene transfer, killing and replication ability of Ads in various non-human cell lines (hamster, murine, rat, bovine, canine and pig) and showed that among tested cell lines only porcine cells were supportive for binding and active replication of Ad3-retargeted vectors. Of note, Ad5/Ad3-chimeric virus showed stronger replication compared to Ad5 in both human and porcine cell lines. Next, we intravenously injected pigs with a high dose (3×10^{12} vp) of each OAd (Ad5 and Ad5/Ad3). Liver biopsies were obtained on days 1, 2, and 4, and necropsy was performed on day 7. The complete necropsy showed no difference compared to the control. There were no abnormalities in blood parameters and serum chemistry. The blood count including the number of platelets was not affected. AST and LDH, as an indication of both liver and lung damage, showed mild elevation on day 4 and returned to normal at day 7. Liver specific ALT was normal in all pigs and the liver damage marker SDH showed mild elevation at day 7 only in the Ad5 group. Remarkably, viral DNA and replication-dependent luciferase expression were observed in lungs and spleen of Ad-injected pigs, while none was detected in liver samples throughout the study. The detailed histology exam of tissue sections revealed signs of Ad-induced interstitial pneumonia in lungs, but no changes in liver. There were no unusual necropsy observations or microscopic lesions in the liver samples. These data contradict the commonly known observations in mouse models, where subsequent to intravenous injections Ad generally targets the liver. In summary, these studies provide a rationale for the use of pigs to study performance of OAds including the vectors with altered tropism. The studies also question the reliability of a mouse model for analyses of OAd biodistribution and liver toxicity. Therefore, we are currently investigating OAd biodistribution, liver uptake and immune response in pigs at earlier time points after systemic OAd administration (1, 6, 24 hours). We have also started developing a swine model of pancreatic cancer by producing a genetically-engineered pig with inducible human KRAS and TP53 mutations. We hope this pig model will foster clinical translation of oncolytic viruses.

593. MEK Kinase Inhibitor, Trametinib, Suppresses Macrophage-Secreted TNF-α on Oncolytic HSV-1 Therapy, Enhancing Virotherapy

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Purpose: Oncolytic herpes simplex viruse-1 (oHSV) therapy is currently FDA-approved for advanced melanoma and is currently being evaluated in several clinical trials. However, its clinical success has been limited and various combinatorial treatment modalities are being investigated to enhance the efficacy of each treatment. Microglia/macrophage-secreted TNFα reduces oHSV replication through the induction of apoptosis in oHSV-infected tumor cells. In this study, we tested the potential role of trametinib, a highly potent MAPK/ERK kinase (MEK) inhibitor, against oHSV-induced TNFα response in macrophage/microglia, and analyzed the underlying mechanisms. **Experimental Design:** In vitro co-culture assays of infected glioma cells with microglia/macrophage cells treated with/without trametinib (100nM) were used to test the effect of trametinib effect on oHSV replication. Q-PCR and ELISA for TNFα were used to test trametinib-mediated TNFα gene expression and protein secretion. Bone marrow derived primary macrophages from wild type and TNFα-knockout mice were used to evaluate the biologic effects of TNFα on virus replication in the setting of trametinib. Western Blot analysis was used to evaluate the induction of apoptosis. Bioluminescence mice imaging were used to measure changes in virus replication *in vivo*. Intracranial tumor xenografts were utilized to evaluate anti-tumor efficacy. **Results:** Trametinib resulted in a significant dose dependent reduction of TNFα gene expression and protein secretion in microglia (BV2) or macrophage (Raw264.7) cells. A significant reduction of microglia/macrophage derived TNFα (gene expression and protein secretion) was also observed in response to oHSV-infected glioma cells *in vitro* and *in vivo*, resulting in increased oHSV replication. Furthermore, trametinib treated BV2 or Raw264.7 cells significantly inhibited BV2-/Raw264.7-mediated apoptotic cell death in the oHSV-infected glioma cells *in vitro* and *in vivo*. Finally, combination treatment with trametinib and oHSV significantly enhanced murine survival. **Conclusions:** This study provides strong evidence to combine MEK inhibitor and oHSV, which significantly enhance survival in mice bearing intracranial glioma, leading to future clinical testing of oHSV with trametinib in patients.

594. CD133 Infectivity-Selective Oncolytic Adenovirus Effectively Targets Radiation-Resistant Colorectal Cancer Cells

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Mortality from colorectal cancer (CRC) remains high due to recurrent disease. CRC stem cells that overexpress the cell surface marker CD133 contribute to therapeutic resistance, tumor initiation, and decreased survival in patients. The Infectivity Selective Oncolytic Adenovirus (ISOAd) showing selective cancer killing ability at the level of infection is a promising approach to target cells that overexpress CD133. In this study, we investigate the efficacy of targeting radiation-resistant CRC cells using a novel ISOAd that specifically infects CD133 positive cells. Our lab previously reported the isolation of an infectivity selective CD133-targeted oncolytic adenovirus, using high-throughput screening of high-diversity adenovirus-format fiber library. Selective binding of CD133-targeted ISOAd was observed with CD133-expressing 293 cells. In order to establish radiation-resistant cells, two CRC cell lines (LS174T and SW480) were repeatedly exposed to fractionated ionizing radiation and surviving colonies were further propagated. The extent of radiation resistance was quantitated using a clonogenic survival assay in vitro and tumor establishment assay in vivo. Flow cytometry for cell surface CD133 showed 20-fold increase for LS174T cells and 4-fold increase for SW480 cells. Compared to radiation-naïve cells, cytotoxicity was observed in a virus-dose dependent manner by quantitative killing assay after treatment of radiation-resistant CRC cells with CD133-targeted ISOAd that has selective binding and infection of CD133-positive cells. Importantly, the replication of CD133-targeted OAd increased by 2 log-folds in irradiated compared to non-irradiated cells ($p<0.05$), showing that the observed cytotoxicity is likely a result of progressive viral replication. In athymic nude mice, the tumor initiating capability of radiation-naïve and radiation-resistant CRC cells was assessed by tumor formation assay. Treatment of radiation-resistant cells with CD133-targeted ISOAd abolished tumor-forming capacity, compared to radiation-naive cells (5% vs 100% respectively, $p<0.05$). When the antitumor effect of the CD133-targeted ISOAd was analyzed in subcutaneously established tumors, treatment with radiation and CD133-targeted ISOAd significantly reduced tumor growth compared to no treatment and treatment with radiation only (both $p<0.01$). Our ISOAd targeted to CD133 at the level of infection is effective for cytotoxic killing and reduction of tumor formation in radiation resistant CRC cells. In vivo, CD133-targeted ISOAd eliminates tumor formation capability and has an antitumor effect in established tumors. This targeted OAd therapy may be applicable to address therapeutic resistance and prevent the establishment of recurrent colorectal cancer.

595. Novel Group C Oncolytic Adenoviruses Demonstrate Enhanced Oncolytic Activity In Vitro and In Vivo

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Oncolytic adenoviruses (AdV) are an innovative treatment option for many human cancers. Most existing oncolytic AdV are based on human serotype 5 (HAdV-5). Clinical efficacy of HAdV-5 based oncolytic viruses is limited by variable expression levels of coxsackie- and adenovirus receptor (CAR) in tumor cells and insufficient replication rates. Additionally, high prevalence of neutralizing antibodies against HAdV-5 makes it a less suitable candidate for systemic application. Studies have highlighted other human AdV as promising candidates for oncolytic therapy. Thus, development of novel oncolytic AdV based

on additional serotypes may help overcoming these limitations. We further hypothesized that oncolytic efficacy of the candidate viruses can be augmented by expression of RNAi suppressor protein P19 as has been shown previously for HAdV-5. Moreover, we intended to limit viral replication to cancer cells by deleting the pRB binding site in the E1A gene. Here we aim at evaluating novel p19-containing oncolytic AdV as candidates for oncolytic applications in vitro and in vivo. We cloned P19-containing viruses based on HAdV-1, -2, -5 and -6 by a novel seamless recombineering technique. To allow P19 expression from the adenoviral vector genome, the P19 cDNA was fused via an internal ribosome entry site (IRES) to the late fiber gene, generating group C vectors HAdVC- FP19. Then, base pairs 922-947 from the AdV E1A gene were deleted using the same technology. After rescue and upscaling, virus purification using cesium-chloride density-gradient ultracentrifugation was performed. Various cancer cell lines including lung (A549), colon (HCT116, Caco-2), gynecologic (HeLa, MCF7), bone (U-2 OS, Saos-2) and pancreatic carcinoma (MIA Paca-2, PANC-1, AsPC-1) were studied for anticancer activity of HAdV variants in vitro. We observed higher cell lysis capability of HAdV-1,-2 and -6 in most cell lines as compared to HAdV-5 at identical VP/cell ratios, suggesting enhanced oncolytic potential. The oncolytic effect could be enhanced by expression of P19 contained in HAdV-2 and HAdV-6 but not in HAdV-1. Differences in oncolytic effects of HAdV variants were observed in a cell type-dependent manner: HAdV-2 showed high oncolytic activity even at low MOI in HeLa and Caco-2 cells, HAdV-1 in Caco-2 cells. Then, the variants were compared to HAdV-5 and existing oncolytic adenovirus H101 (Oncorine) in a A549 tumor xenograft mouse model. P19-containing HAdV based on HAdV-1, -2, and -6 showed significantly higher anti-tumor potency and significantly longer time to tumor progression compared to Oncorine (H101). This observation was in concordance with in vitro experiments. In total, we believe that oncolytic vectors based on other serotypes than HAdV-5 hold great promise for oncolytic applications and that their effectiveness can be further improved by RNAi-suppression.

596. A Neuregulin-Like Gene Deleted Recombinant Tanapoxvirus Demonstrates Increased Oncolytic Potency and Enhanced Immune-Stimulatory Activity

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Melanoma is one of the most common skin cancers with poor prognosis and survival rate. Oncolytic viruses, with the ability of causing replicative oncolysis and expressing toxic and/or immuno-stimulatory gene products, serve as an appealing addition to the melanoma therapy. Tanapoxvirus (TPV) is potentially a great candidate for melanoma therapy, as it is a benign virus that has exhibited tumor-regression of colon cancer induced in animal models. Herein we show that the TPV-15L protein, a functional mimetic of neuregulin (NRG), exhibits similar effectiveness as NRG in promoting melanoma growth *in vitro*. Therefore, TPV recombinants were generated with either the 15L gene deleted (TPVΔ15L) or with both 15L and the viral thymidine kinase gene (66R) ablated (TPVΔ15LΔ66R). The replication kinetics of TPVΔ15L was similar to that of wtTPV. However, TPVΔ15LΔ66R replicated less efficiently than TPVΔ15L and the parental virus *in vitro*. TPVΔ15L exhibited more robust tumor-reduction efficacy in the melanoma-bearing nude mice than other recombinant TPVs *in vivo*. Our results indicate that the deletion of the 66R gene, but not 15L gene, adversely affects virus replication, and that deletion of 15L (which elevates melanoma proliferation) enhances virus oncolytic efficacy. Interestingly, an antiviral activity, which was identified as interferon-λ1 (IFN-λ1), was secreted in a significantly higher quantity by the cells infected with TPVΔ15L. We show that IFN-λ exhibits a more pronounced anti-proliferative effect in melanoma than IFNα and IFNβ *in vitro*. Further, we demonstrate that anti-IFN-λ1 exerts an inhibitory effect on melanoma cell apoptosis caused by TPVΔ15L infection, which indicates that TPVΔ15L regresses melanoma growth partially through inducing IFN-λ1 release. Taken together, our results demonstrate that TPVΔ15L should be explored further for melanoma oncolytic virotherapy.

597. Oncolytic Virotherapy with Simultanously Enhanced Oncolytic Activity and Safety

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Oncolytic virotherapy (OV) is a promising approach in cancer treatment. However, enhancing the clinical efficacy of OV without compromising the safety remains a challenge. To improve OV's efficacy and safety simultaneously, our group have developed the following three strategies for oncolytic HSV-1s: 1) to generate oHSVs with better controlled tumor specific viral replication without deleting viral genes. We have shown that by combining tumor specific translation and transcriptional regulatory elements to control the expression of a viral essential gene (TTDRoHSV), replication of the OV can be restricted in tumor cells without needing to delete any viral native gene, which allows much higher oncolytic activity in tumor mass; 2) to sensitize tumor cells to OV therapy by stimulating host anti-cancer responses, which can be achieved by administrating small molecules that facilitates infected tumor apoptosis and releasing immune stimulating factors expressed by the OVs; 3) to facilitate viral dissemination in tumor mass by overcoming the OV-nonpermissive tumor microenvironment. The latter is formed mainly by tumor associated non-tumor cells, such as macrophages. We found that, by inhibiting STATs pathways, the tumor associated HSV nonpermissive macrophages can be turned into permissive, which facilitates oHSV dissemination in tumor mass with increased efficacy in vivo.

598. Breaking Multiple Mechanisms of Resistance of Pancreatic Cancer Cells to Oncolytic Vesicular Stomatitis Virus

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Vesicular stomatitis virus (VSV) based recombinant viruses, such as VSV-ΔM51, are effective oncolytic viruses (OVs) against a majority of tested human pancreatic ductal adenocarcinoma (PDAC) cell lines. However, some PDAC cell lines are highly resistant to VSV-ΔM51, at least in part due to the upregulated type I interferon (IFN) signaling.

Ruxolitinib or other JAK1/2 inhibitors strongly stimulate VSV-ΔM51 replication and oncolysis in all resistant cell lines, however, only partially restore their permissiveness to VSV-ΔM51. Here, we conducted a series of experiments to determine if VSV-ΔM51 attachment was inhibited in resistant PDAC cell lines. Our data showed a dramatically weaker attachment of VSV-ΔM51 to HPAF-II, the most resistant PDAC cell line. The weaker VSV attachment to HPAF-II cells correlated with lower expression levels and dramatically lower activity of the low-density lipoprotein receptor (LDLR), one of the cell surface receptors for VSV. Treatment of cells with various statins strongly increased LDLR expression levels, but VSV-ΔM51/statin combination treatments did not improve VSV-ΔM51 attachment or replication. Therefore, we tested various conditions to improve LDLR-independent attachment of VSV to HPAF-II cells. We show that VSV attachment can be dramatically improved by treating cells with polycations. Moreover, we successfully used a novel approach to break resistance of HPAF-II to VSV-ΔM51 by combining it with ruxolitinib and polycations, thus simultaneously improving VSV attachment and inhibiting IFN signaling.

599. Biosafety Reclassification of Tumor-Bearing Mouse Model Following Adenovirus Type 5 Administration

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Objective: Adenovirus is a known human pathogen, and is designated by the NIH as a Biosafety Level 2 organism. The goal of this study was to clarify a time point at which replication competent adenovirus type 5, administered to a mouse model, ceases to pose an infectious risk to laboratory personnel through viral replication and/or viral shedding. Determination of this time point allows the biosafety containment to be downgraded from Animal Biosafety Level 2 (ABSL2) to Animal Biosafety Level 1 (ABSL1). Additionally, we determined if viral replication and shedding in a mouse tumor model is affected by a human-origin tumor burden. **Methods:** SCID mice were divided into two groups: one remaining naïve until virus injection, the other bearing bilateral, subcutaneous human pancreatic Panc1 tumors. When tumors measured 6-9mm in diameter, all animals were intravenously injected with $5x10^{10}$ vp of Ad5WT, a wild-type human adenovirus. Dermal, buccal, urine, and fecal samples were taken immediately prior to Ad5Wt administration, and thereafter on Days 1, 3, and 7. Samples were processed through an appropriate QIAGEN DNA extraction kit, and viral copy number was quantified by qPCR. Additionally, samples containing high viral copy numbers as indicated by qPCR were selected for further assessment by Plaque Forming Unit (PFU) assay to assess the infective ability of recovered virus. As a positive control, a sterile swab was dipped in a solution of $1x10^4$ vp/mL of Ad5WT and similarly processed, and applied to cellular culture. **Results:** Adenoviral copies, as quantitated by qPCR, were found in most samples on Day 1 after viral administration, but quickly reduced to background levels. By Day 3, the buccal and dermal samples had viral copy counts almost equal to the Day 0 baseline levels. Meanwhile, the urine samples did not increase in viral count on Day 1, but displayed peak copy numbers on Day 3. However, the viral concentration of the Day 3 urine samples was far below that of the Day 1 dermal samples, and similar to the Day 1 Buccal samples. While qPCR indicated several samples containing high viral counts on Days 1 and 3 (buccal and urine, respectively), no adenoviral plaques were present in the PFU assays corresponding to these highest qPCR results. These data confirm no functionally infective viral particles are present in the collected samples, despite some elevation in viral copy numbers obtained by qPCR. The animals bearing Panc1 subcutaneous tumors showed no difference in virus shedding from the tumor free animal, nor a difference in viability of recovered virus. **Conclusion:** The lack of biological activity as determined by PFU assay supports the reclassification of adenoviral vector mouse models from ABSL2 to ABSL1 at 72 hours after viral administration. The presence of subcutaneous human cancer tissue has no effect on viral viability or copy number count. Our study, thus, provides pertinent information to Institutional Biosafety Committees regarding risk management of adenoviral vector use in animal models. The reclassification of biosafety hazard level will reduce costs associated with ABSL2 housing protocols, and allow increased flexibility with animal handling, transportation, and experimental treatments subsequent to virus administration.

600. Expression and Characterization of Different Human Papillomavirus Types L1 Capsid Protein in Pichia Pastoris

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Human papillomavirus (HPV) infection is the most common sexually transmitted disease in the world and is related to the development of cervical cancer. The vaccine strategies against HPV have shown that virus-like particles (VLPs) made from the major capsid protein (L1) can induce efficient production of antibodies, so several researches focus on the expression of HPV L1 protein in heterologous systems to obtain VLPs as HPV vaccine candidates. The methylotrophic yeast *Pichia pastoris* is an efficient and inexpensive expression system and its production of high levels of heterologous proteins is stable. Here, we describe the expression, purification and characterization of recombinant HPV6, 11, 16, 18 L1 proteins produced in *P. pastoris*. The codon-optimized HPV L1 genes of four different HPV types 6, 11, 16, 18 was cloned into pPink-HC expression vector under the regulation of a methanol-inducible promoter, meanwhile we designed different Kozak sequence (ACC and GAAACG) for these L1 genes to compare the yield and morphology of VLPs. The VLPs produced in *P. pastoris* were subjected to Polyethylene Glycol (PEG) precipitation and sucrose density gradient centrifugations. The purified VLPs were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transmission electron microscopy. The results showed these VLPs of four types were of highly purity with diameter of approximately 50nm. What's more, the HPV 16 and 18 cassettes with Kozak sequence ACC generated more uniform particles than cassettes with GAAACG. The display of conformational epitopes on the VLPs surface was demonstrated by hemagglutination assays. To test the immunogenicity of recombinant L1 proteins, we immunized BALB/c mice with single or tetravalent VLPs respectively, and the HPV antibody titers were measured by ELISA at 2 weeks post vaccination. The high serum antibody titers were observed significantly in all groups while tetravalent VLPs vaccination showed higher titers than single VLPs. Our results prove that the L1 protein of different HPV types expressed

in *P. pastoris* fold properly as evidenced by assembly into VLPs and induction of humoral immunity in mice. And this work can provide an alternative platform for the production of low-cost HPV vaccines.

601. Use of Oncolytic Adenovirus Expressing IFNα to Improve IFN Therapy Against Pancreatic Cancer

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Aside from surgery, there is no effective therapy against pancreatic adenocarcinoma (PDAC). Late diagnosis and high recurrence results in post-diagnostic survival of 6 months and 5-year survival of 7%. Notably, Phase II trials using systemic IFN- α (IFN), radiation, and combination of 5-FU + Cisplatin (CDDP) following surgery reported increase in PDAC 2-year survival by 35%, and in the 5-year survival by 21%. Trials adding Gemcitabine (GEM) to IFN treatment also reported increase in 2-year survival by 56%. Despite promising results, IFN therapy drawbacks included high IFN systemic toxicity, and low IFN levels in tumors. IFN toxicity resulted in high patient dropout, and as IFN potentiates chemoradiation killing effect in cancer cells, low IFN levels in tumors hampered therapy effectiveness. Aiming to improve efficacy and tolerability of IFN therapy, we developed an oncolytic adenovirus expressing human IFN (OAd-IFN). Vector has Ad5/3 fiber modification and overexpresses Adenoviral Death Protein, respectively contributing to increased infectivity and oncolysis. As Cox-2 is up-regulation in PDAC, this promoter was included upstream of Adenovirus (Ad) E1 region restricting vector replication to PDAC. IFN transgene was placed in the Ad E3 region permitting replication dependent IFN expression. In vivo testing of OAd-IFN effectiveness in treatments resembling IFN therapy (IFN-chemoradiation) was done in nude mice bearing human PDAC cell xenografts. We tested OAd-IFN efficiency in combination with chemoradiation using Colony Formation Assay (CFA), as it better represents effect of radiation in vitro. CFA in Mia Paca and S2013 human PDAC cells showed that OAd-IFN increased cell killing when combined with chemotherapy (5-FU, GEM), radiation, and chemoradiation. Use of OAd-IFN with CDDP + 5-FU does not improve cell killing, but combinations of OAd-IFN with CDDP+5-FU and radiation are highly cytotoxic. Comparison between OAd-IFN and control vector not expressing IFN (OAd-LUC) indicated that IFN expressed by OAd-IFN potentiated PDAC cell death by chemotherapy (5-FU, GEM, 5-FU + CDDP), radiation, and chemoradiation in PDAC cells. Combination index (CI) analysis demonstrated OAd-IFN use with chemotherapy (5-FU and GEM), radiation, and chemoradiation are highly synergistic. CI of treatments with OAd-IFN and CDDP+5-FU were shown to be antagonistic, but addition of radiation to treatment (OAd-IFN with CDDP + 5-FU and radiation) resulted in the strongest synergistic interaction amid all combinations tested in vitro. In vivo studies in mice bearing PDAC tumors showed that treatments of OAd-IFN with radiation, and OAd-IFN with CDDP + 5-FU and radiation equally inhibited tumor growth, and use of OAd-IFN with radiation showed sustained tumor growth inhibition and longer survival. While use of OAd-IFN with CDDP + 5-FU and radiation effectively inhibited tumor growth, groups treated with OAd-IFN and CDDP + 5-FU did not inhibited tumor growth. This may suggest that use of CDDP + 5-FU affects viral replication. While conclusion requires further analyses, it is possible that in vitro antagonism predicted for OAd-IFN and CDDP+5-FU combination is also reflected in vivo. Thus, to improve efficacy of OAd-IFN treatment with chemoradiation separated administration of virus and CDDP + 5-FU might be required. Our data suggests that OAd-IFN has great potential to be developed in an improved IFN therapy to treat PDAC. Oncolytic effect of OAd expressing high levels of IFN intratumorally paired with strong synergism between virus, chemotherapy, and radiation suggests OAd-IFN can augment IFN therapy efficacy while maintaining minimal toxicity. As IFN therapy was one of the few treatments known to increased long term survival in PDAC, use of OAd-IFN to develop this therapy can greatly contribute to progress in PDAC treatment.

602. Interleukin-2-Expressing Oncolytic Tanapoxvirus: A Promising Strategy for Melanoma Immuno-Virotherapy

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Oncolytic viruses (OVs), which preferentially infect cancer cells and induce host anti-tumor immune responses, have emerged as an effective melanoma therapy. Tanapoxvirus (TPV), which possesses a large genome and causes mild self-limiting disease in humans, is potentially an ideal OV candidate. Interleukin-2 (IL-2), a T-cell growth factor, plays a critical role in activating T cells, natural killer (NK) cells and macrophages in both the innate and adaptive immune systems. In this study, a recombinant TPV expressing mouse IL-2 (TPVΔ66R/mIL-2) was generated, where the viral thymidine kinase (TK) gene (66R) was replaced with the mIL-2 transgene. In cell culture, expression of IL-2 attenuated virus replication of not only TPVΔ66R/mIL-2, but also TPVGFP when co-infecting with TPVΔ66R/mIL-2. Further, we demonstrate that IL-2 inhibits virus replication through intracellular components and without activating the interferon-signaling pathway. The anti-tumor potential of TPVΔ66R/mIL-2 was studied in athymic nude mice (T cell deficient animals) burdening human melanoma xenografts. Introduction of mIL-2 into TPV remarkably increased its anti-tumor activity, resulting in a more significant tumor regression than with wild-type (wt) TPV and TPVΔ66R. On average the tumor volume increased by 4.49%/day in the mice with mock treatment but only by 0.06%/day in the TPVΔ66R/mIL-2-treated mice. In contrast, mean tumor growth rate in the wtTPV- or TPVΔ66R- treated mice did not significantly differ from that in the mice treated with mock injection. Histopathological observations showed that treatment with TPVΔ66R/mIL-2 resulted in more extensive tumor cell degeneration and increased mononuclear cells accumulation (some of which had a macrophage phenotype-F4/80), in comparison to treatment with the other TPVs. These data provide compelling evidence that IL-2 induces a host anti-tumor innate immune response for tumor regression in the absence of mature T cells. Taken together, our results suggest that TPVΔ66R/mIL-2 is a promising immuno-virotherapy for melanoma, and that IL-2 expression results in an overall increase of therapeutic efficacy despite its viral inhibitory effects.

Cancer-Targeted Gene and Cell Therapy II

603. Exploring the Activity Landscape of Antibody-Coupled T-Cell Receptors

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Redirected T-cell therapy is a promising option for cancer treatment because of its potential to specifically target tumor cells while sparing normal tissues. Recent results of clinical trials with infusions of chimeric receptor-expressing autologous T lymphocytes provide compelling evidence of their clinical potential. We have demonstrated a more universal approach to T-cell therapy by engineering T-cells to express an Antibody Coupled T-cell Receptor (ACTR) comprised of the ectodomain of CD16 fused to the 4-1BB costimulatory and CD3-zeta signaling domains. These ACTR-T-cells exert antibody-dependent cell-mediated cytotoxicity, a function otherwise physiologically limited to CD16-expressing natural killer cells and macrophages.

We sought to explore the activity landscape of ACTR to generate variants with optimized function for use in challenging therapeutic environments. Over 90 different domain-modified variant ACTRs were generated and evaluated in assays in vitro. Short-term cytotoxicity assays proved to be non-differentiating, necessitating evaluation of multiple activity measures to understand diversity in ACTR function. The activity of ACTR variants was differentiated in cytokine release and in 7-day proliferation assays in the presence of target cells and target-specific antibody. A repeated-stimulation assay was also developed in which ACTR T-cells were stimulated with antibody and target cells every 3 - 4 days over the course of 14 days and their proliferative ability was measured in an effort to simulate longer-term T-cell survival.

ACTR variants were identified with varied activity profiles. For example, ACTR variants lacking a CD8 hinge region out-performed their hinged counterparts, suggesting that synapse distance plays a role in activity. Varying the ACTR costimulatory domain also impacted activity. ACTR variants with 4-1BB, CD28, and ICOS costimulatory domains showed higher IL2 production and greater target/antibody-dependent proliferation than variants with CD27 and OX40 costimulatory domains. In repeated stimulation assays, a surrogate measure of greater T-cell survival, the CD28 variant maintained a higher ability to proliferate throughout the course of the assay than the 4-1BB and ICOS variants. This is in contrast to published reports with CAR variants that demonstrate superior long-term in vitro survival for 4-1BB-bearing variants. These results suggest that the ACTR platform may be more tolerant than CAR to the stronger costimulation mediated by CD28 without concomitant T-cell exhaustion and loss of T-cell fitness over time. Lastly, we have identified ACTR variants that demonstrate robust anti-tumor activity in stringent models in vivo.

We have screened a large number of ACTR variants employing a multi-assay approach to identify ACTR variants with varied activities. The diversity of readouts and mix of short- and long-term in vitro assays proved essential to dissect differences in activities amongst the ACTR variants. This approach can be further applied to evaluate large numbers of chimeric receptors in T-cells to identify candidate molecules with desired properties for further testing in vitro and in vivo.

604. Engineering Ex Vivo Serum-Free Expanded γδ T Cells for the Treatment of Neuroblastoma

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Bridging of the recognition and cytotoxic functions of cell-mediated adaptive and innate immunity continues to be an attractive application of anti-cancer cellular immunotherapeutics.

The effectiveness of αβ T cells can be increased through genetic engineering with chimeric antigen receptors (CARs), which allows the modified cells to bypass MHC-restricted recognition while still activating through CD3ζ signaling. Unlike αβ T cells, γδ T cells i) are innate immune cells, ii) do not require antigen priming, iii) have intrinsic anti-tumorigenicity, and iv) induce tumor killing via recognition of stress antigens and through mechanisms of antibody-dependent cellular cytotoxicity (ADCC). Because less than 30% of resting γδ T cells express CD16 (FCγ receptor III), a major receptor responsible for ADCC, enriching the CD16-expressing γδ T cell population from peripheral blood mononuclear cells (PBMC) presents a reasonable option to enhance ADCC. Using RNAseq and flow cytometry, we show that *ex vivo* expansion of γδ T cell from normal donors in serum-free conditions with a bisphosphonate and IL-2 increases surface expression of CD16 to greater than 90% within 14 days of expansion. Additionally, we have now tested the expansion potential of γδ T cells from PBMCs of neuroblastoma patients, and we show γδ T cells can be expanded to >75% of the total population with 97% of the γδ T cells expressing CD16. These cells maintain cytotoxicity against 8 neuroblastoma cell lines, including several aggressive cell lines with *MYCN* amplification. Further, combining patient-expanded γδ T cells that express CD16 with a GD2 specific antibody, Dinituximab, induces 30% increased neuroblastoma cell death compared to γδ T cells alone, where the antibody-independent killing in a 5:1 effector to target ratio was in the range of 10-40% and with Dinituximab the range increased to 25-52%. GD2 surface expression on neuroblastoma cell lines ranged from 12-98% and antibody-dependent cell killing correlated with GD2 expression. Interestingly, there were fluctuations in cytotoxicity among subjects, which varied by as much as 50% for some cell lines, suggesting that expanded γδ T cells from neuroblastoma patients may respond differently depending on the patients' disease profile. In addition, to introduce adaptive immune killing mechanisms into these innate cells we utilized various CAR constructs. An anti-GD2 single chain variable fragment (scFv) CAR was engineered and cloned into a lentiviral expression vector. Transduction on days 6-8 resulted in approximately 20% genetic modification, which increased neuroblastoma cell killing four-fold compared to unmodified γδ T cells, demonstrating enhanced killing by CAR-modified γδ T cells. Overall, these data show that a combination of serum free expansion,

increased CD16 expression, and genetic modification with an anti-GD2 CAR enhances γδ T cell-mediated neuroblastoma cell killing, thus providing a safety enhancement of immunotherapy for neuroblastoma.

605. Aptamer-Based STAT3 RNAi Targeted Therapy in GBM

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GBM (glioblastoma multiforme) is the most common primary brain tumour and one of the most lethal of all cancers, despite the advancements in surgical and therapeutic approaches the prognosis remains dismal. A minor cell population, constituted of multipotent tumor-initiating stem-like cells (GSCs), has been recently implicated in GBM recurrence and resistance to conventional treatments. Thus, the identification of new strategies able to target GBM would represent a great challenge for patients management. Oligonucleotide-based strategy revealed a great potential as anti-cancer therapeutics, but the development of safe, effective and selective approaches for their delivery remains a challenge. In this regard, aptamers exhibit many desirable properties for tumor-targeted drug delivery, such as ease of selection and synthesis, high binding affinity and specificity, low immunogenicity, and versatile synthetic accessibility. We have selected and characterized two aptamers (GL21.T and Gint4.T) that bind to, and inhibit the receptor tyrosine kinases Axl and PDGFRβ and able to selectively deliver miRNA/antimiR molecules to GBM cells. Notably, we showed that both aptamers as either single molecules or conjugates are transported through an *in vitro* blood-brain barrier (BBB) model, thus revealing a means for the selective and effective treatment of glioma tumours and GSCs. Here, we took advantage from the developed strategy, for the targeted delivery of small interfering RNA antagonizing the signal transducer and activator of transcription-3 (STAT3) that has been reported as a key regulator of the highly aggressive glioblastoma subtype and implicated in the formation of the GSC population. We generated novel aptamer-STAT3 conjugates and demonstrated the selective delivery of the siRNA and the efficient silencing of STAT3 in aptamer target positive GBM cells. Importantly, conjugate treatment specifically results in reduced glioblastoma cell viability, thus indicating this molecule as a novel tool with great translational potential for glioma-targeted therapy.

606. Oncoleaking Suicidal *Clostridium perfringens* Enterotoxin Gene Therapy for Pancreatic Cancer

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Bacterial toxins have evolved to an effective therapeutic option for cancer therapy and their antitumoral potential has been demonstrated in numerous studies. *Clostridium perfringens* Enterotoxin (CPE), produced by the anaerobic *Clostridium perfringes* bacteria, is a pore forming toxin with a selective, receptor-dependent cytotoxicity. The tight junction proteins claudin-3 and claudin-4, known to be high-affine CPE receptors, are highly upregulated in several human epithelial cancers such as breast, colon, ovarian and pancreatic cancer. CPE binding to its receptor triggers membrane pore complex formation, which leads to rapid cell death.

Here we aimed at evaluation of an efficient non-viral therapeutic approach to treat claudin-3 and -4 overexpressing pancreatic cancer using a translation optimized CPE expressing vector (optCPE) *in vitro* and *in vivo*.

We investigated the sensitivity of human pancreatic cancer cell lines and more interestingly patient derived pancreatic cancer xenograft (PDX) derived cells for treatment with recombinant CPE (recCPE) as well as by optCPE gene transfer. Claudin-3 and / or -4 overexpressing cancer cells revealed high sensitivity towards both, recCPE treatment and optCPE gene transfer. Particularly for optCPE toxicity of up to 90% was observed 72h after gene transfer. Further, we demonstrated a positive correlation between cytotoxic activity of CPE and level of claudin expression. The optCPE gene transfer led to rapid cytotoxic effects such as massive membrane disruption, cell as well as nuclear disintegration. This supports the targeted CPE toxicity, which specifically eradicates claudin-3 and / or -4 overexpressing pancreatic cancer cells. Selectivity of CPE action was further proven by use of isogenic cell lines with forced or downregulated claudin expression. The non-viral intratumoral *in vivo* gene transfer of optCPE led to reduced tumor viability associated with tumor necrosis and inhibition of tumor growth in pancreatic cell line xenotransplant and PDX bearing mice compared to the respective control groups.

This study emphasizes the great potential of this targeted CPE mediated oncoleaking suicidal gene therapy of claudin-3 and / or -4 overexpressing pancreatic tumors.

607. Balance of Anti CD123 CAR Binding Affinity and Density for Acute Myeloid Leukemia

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Chimeric Antigen Receptors (CARs)-redirected T lymphocytes are a promising immunotherapeutic approach, nowadays object of accurate preclinical evaluation also for Acute Myeloid Leukemia (AML) targeting. We recently developed a CAR against the CD123 antigen, found to be over-expressed on AML blasts and leukemic stem cells (LSCs). However, the potential recognition of low CD123-positive healthy tissues, through the so called on target off organ effect, limits the safe clinical employment of CAR-T cells. In search for an optimization of this strategy, we evaluated the effect of several variables implicated in the CAR design, known to modulate CAR T-cell functional profiles in a context-dependent manner, such as CAR binding affinity, CAR expression and target antigen density. Therefore, we developed a novel integrated model for the functional screening of in silico-selected CAR affinity mutants (CAM), starting from predicted antibody binding properties, that displayed different binding affinities: CAM-H1 (High Affinity 1), CAM-H2, which maintained the same binding affinity of the wild type (wt) anti-CD123 CAR (10-9 Molar), CAM-M (Medium affinity) and CAM-L (Low affinity), displaying a 10 and 100 fold affinity reduction, respectively. The in vitro functional characterization of Cytokine-Induced Killer (CIK) effector cells genetically modified with the CAMs has allowed to define both lytic and activation antigen thresholds showing that, while the early cytotoxic activity is not affected neither by CAR expression nor by CAR affinity tuning, the CAR expression represents the main variable impairing later effector functions. All these variables are essential for a further clinical translation of this approach, and the lowest affinity mutant could represent the one with an affinity threshold granting a proper balance between safety and efficacy profiles, below which the antileukemic efficacy could be impaired.

608. An Inhibitor for the MDM2-p53 Interaction Produces Synergistic Cytotoxicity with Oncolytic Adenoviruses on Mesothelioma with Wild-Type p53 Genotype

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A majority of clinical specimens from mesothelioma does not express p14 or p16 mostly due to homologous deletion of the INK4A/ARF region and partly due to methylation of the transcriptional regulatory regions, whereas the p53 genotype of the cases is wild-type. Recent whole exon sequence data also demonstrated a high frequency of the deletion which encodes the p14 and the p16 genes in mesothelioma specimens. Consequently, the majority of mesothelioma is functionally defective of the p53 pathways since the p14 deficit augments a MDM2 activity that facilitates p53 degradations through ubiquitination processes. We examined whether activation of the endogenous p53 pathway with a chemical that blocked p53 degradation through inhibiting the MDM2-p53 interaction achieved cytotoxic effects on mesothelioma with the wild-type p53 genotype, and further investigated a possible combinatory use with oncolytic adenoviruses (Ad) which enhanced endogenous p53 levels. Nutlin-3a and RITA (reactivation of p53 and induction of tumor cell apoptosis) inhibited the interactions and subsequently produced cytotoxicity on mesothelioma depending on the p53 genotype. The agents increased phosphorylation of p53, activated p53-mediated cell cycle arrest and induced apoptosis thereafter. Ad defective of the E1B55 kDa gene (Ad-E1B) induced cell death and augmented endogenous p53 levels in mesothelioma with the wild-type p53 since E1 activated p53 but E1B55 kDa molecules degraded p53. Ad-E1B-mediated cytotoxicity was thereby produced by the viral replications and partly attributable to activation of the p53 downstream pathway. We then tested a possible combinatory use of the inhibitors and Ad-E1B, and found that the combination produced synergistic cytotoxicity. The combination inhibited cell growth and increased sub-G1 fractions greater than an individual agent. Cells treated with the inhibitor increased expression levels of MDM2 and p21, p53 target molecules, but those treated with the combination decreased these levels to facilitate cell death. In contrast, the combination induced phosphorylation of p53 and H2AX, and cleavage of PARP and caspase-3 greater than Ad-E1B alone. Nevertheless, expression levels of Atg 5, Beclin 1, or LC3A/B II remained unchanged in the combination. These data collectively suggested that replication-competent Ad-E1B produced synergistic cytotoxic effects to a majority of mesothelioma with an inhibitor for MDM2-p53 by augmenting DNA damages and subsequent p53-mediated apoptosis.

609. Continuous Administration of Valganciclovir Improves Suicide Gene Therapy for GBM Treatment

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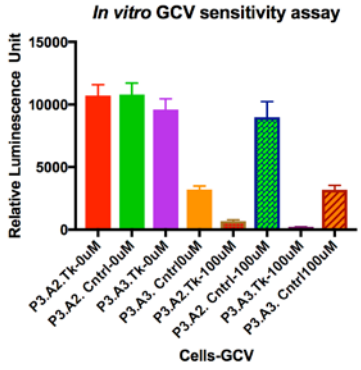
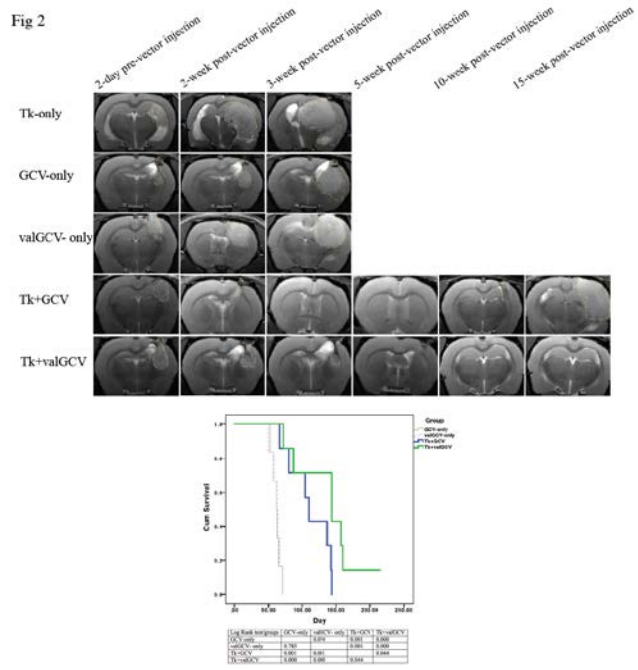
Lentiviral vector mediated herpes simplex virus thymidine kinase (HSV-Tk)/ ganciclovir (GCV) therapy is a very promising therapeutic option for the treatment of glioblastoma (GBM). Although this therapy leads to complete remission of GBM in an orthotopic PDX model, recurrent tumors are observed which contain a fraction of tk-GFP$^+$ cells surviving 3 weeks of prodrug administration. We sorted tk-GFP$^+$ glioma cells from the recurrent tumors and observed that the cells are less proliferative and retain sensitivity to GCV (Figure 1). Thus, we showed that short-term prodrug delivery- used in experimental studies and clinical gene therapy trials fails to eliminate a fraction of glioma cells, which are slow proliferating. We hypothesized that a longer period of prodrug administration would provide an enhanced therapeutic effect. As long-term prodrug we used valganciclovir (valGCV), which is similar to GCV, but tailored for oral administration. Prolonged administration of valganciclovir (valGCV) resulted in a significant survival advantage compared to short-term (3 weeks) GCV application (Figure 2) . Nonetheless, the majority of animals treated with valGCV also developed recurrent tumors. These tumors were more invasive compared to the primary tumors and showed significant upregulation of the epidermal growth factor receptor (EGFR). We are currently investigating signaling pathways upstream and downstream of EGFR. Our results warrant a treatment combination of tk/ValGCV gene therapy with EGFR inhibitors, which we are currently investigating.

Fig 1



P3.A2: Recurrent P3 PDX neurospheroids salvaged from from Animal 2
P3.A3: Recurrent P3 PDX neurospheroids salvaged from from Animal 3

Fig 2



610. Targeting of Pyruvate Kinase M2 (PKM2) Gene Induces Subcellular Compartmentalization of PKM2 and Characterizes a Therapeutic Response *In Vitro* and *In Vivo* Mouse Models of Human Non-Small-Cell Lung Cancer

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Previously, we have demonstrated that the M2 isoform of pyruvate kinase (PKM2), an alternatively spliced variant is one of the 97 genes over-expressed in patient-derived non-small-cell lung cancer (NSCLC) cell lines. Since PKM2 promotes cancer cell-specific glucose metabolism known as aerobic glycolysis or the Warburg effect, we studied the effect of targeting this glycolytic enzyme using gene silencing shRNA (*shRNA-PKM2*) or treatment with a small molecule inhibitor of PKM2 (SMI) and characterized the biological response. Both methods of PKM2 targeting significantly reduced mRNA expression of PKM2, PKM2 activity, cell viability, and colony formation of NSCLC cells in a concentration dependent manner ($P<0.001$ compared to controls). Interestingly, both methods of targeting resulted in a significant decrease in cytosolic localization of PKM2 and increase in nuclear compartment ($P<0.0012$) compared to untreated cell cultures *in vitro*. Next, we developed human xenografts in athymic nude mice by implanting fast growing H1299 and slow growing H358 NSCLC cell lines and gave intratumoral injections of different doses of SMI and monitored tumor growth. We also developed xenografts

from H1299 or H358 cell lines after *shRNA-PKM2* knockdown. Both approaches showed a moderate anti-tumor effect in mice, which resulted in complete, partial or no response to PKM2 targeted therapy. By immunohistochemistry analysis, we observed > 70% reduction in cytoplasmic PKM2 with low or undetectable nuclear PKM2 expression in regressing tumors compared to placebo treated control tumors. In contrast, non-regressing tumors showed an opposite trend in which nuclear PKM2 expression was higher (~38%) compared to cytoplasmic PKM2 (~2.86%). Our results indicate a novel function of PKM2 in the cytoplasm and nuclei of NSCLC tumors, which may characterize the therapeutic resistance to anti-PKM2 therapy in pre-clinical models of human NSCLC. Based on these data, we believe that subcellular localization of PKM2 may serve as a useful biomarker of therapeutic response in experimental and investigational studies involving NSCLC.

611. Adaptation of Vectors and Drug-Inducible Systems for Controlled Expression of Transgenes in the Tumor Microenvironment

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Many biological therapies against cancer are based on recombinant proteins such as antibodies, cytokines, etc. Extensive work in animal models and clinical trials validate the biological efffect of these agents. However, clinical success is often limited by inappropriate biodistribution and pharmacokinetics of the proteins when they are administered systemically. Local injection of gene therapy (GT) vectors encoding these proteins is a promising alternative. The ideal vector should allow long-term, controlled expression of the transgene after a single intratumoral administration.

We describe the optimization of fully humanized mifepristone-inducible systems with the ability to achieve tight control of reporter gene expression (luciferase) in a variety of cancer cell lines. Representative expression cassettes containing the transactivator under the control of different constitutive promoters were incorporated into High-Capacity adenoviral vectors. Functional analysis of vectors was performed by in vivo bioluminiscence in C57BL/6 mice treated with a single intravenous injection. The results indicated that the elongation factor 1-alpah promoter achieved the best induction ratio in response to mifepristone and was functional after several cycles of activation. Further improvements in the vector incldude capsid modifications to favor transduction of stromal cells in the tumor. The impact of these chagnes in the biodistribution and stability of transgene expression will be analyzed in orthotopic pancreatic cancer tumors in mice.

612. Evaluating the Quality of Cell Counting Methods for Autologous Cell Therapy Applications Using Experimental Design and Statistical Analysis

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Advanced therapies including cell therapy and gene therapy utilize cells as the therapeutic product. Cell count and viability are important metrics for product development, dosing, and quality control, but cell enumeration involves many sources of variability. In addition, many measurement platforms and instruments have been developed for cell counting in recent years, and discrepancies between measurements obtained using different counting platforms present a challenge in selecting an appropriate cell counting method. In our previous work, we have developed an experimental design and statistical analysis to help assess the quality of a cell counting measurement. The experimental design uses a dilution series study with replicate samples as well as procedures to evaluate pipetting error and control for operator and temporal bias. The statistical analysis generates a set of metrics for evaluating measurement quality in terms of proportionality and precision. In this design, a proportional response to dilution fraction serves as an internal control, where deviation from a proportional response is indicative of a systematic bias or random variation in the measurement process.

This work aims to apply the general experimental design and statistical analysis to achieve better quantification of cell counting for dosing of autologous cellular products. Dosing of these cellular products is generally based on the quantity of cells to be delivered (e.g., number of cells per kilogram of body weight). Errors in cell counting can impact treatment safety and efficacy. To effectively determine the appropriate dose, cell counting measurements should have sufficient precision and robustness. Autologous cellular products present additional challenges for high confidence counting due to limited cell quantity (particularly for measurements) as well as large donor variability and an associated large range of sample properties.

Here we evaluate three cell counting methods for the case of autologous cell therapy dosing to enable the selection of an appropriate cell counting method. Specifically, we assess use of: manual hemocytometer using C-chip slides, and two automated counting methods: Cellometer A2000, an image based analyzer and Advia 120, a flow cytometry based hematology analyzer. We designed counting experiments to account for limited sample availability and variability in cell sample properties from different donors. Preliminary results for the cell samples examined suggest that the Advia 120 measurement system has greater proportionality and precision for total WBC count compared to the other counting methods. For viability analysis, the Cellometer A2000 demonstrated lower variability in % viability across dilution fractions compared to the hemocytometer method. We will show the quality of cell count and cell viability measurements from different methods. In addition, results collected from different donors will be examined to evaluate the robustness of conclusions to sample variability. These efforts support ongoing ISO efforts to develop a package of cell counting standards using this and other strategies.

613. A Comprehensive Analysis of Clinical Trials Assessing Chimeric Antigen Receptor (CAR) Modified T Cells

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Chimeric antigen receptors (CARs) are artificial surface receptors that can be introduced into T-cells to redirect them specifically towards the killing of cancer cells. Recent success stories of cancer therapy with CAR modified T-cells have raised enormous expectations to cure severely ill patients. In order to get an idea about how an ideal CAR T cell and its application should look like, we have systematically gathered all available data about the clinical trials performed so far world-wide and analyzed these for parameters such as type of targeted antigen and indication, the molecular design of the CAR, efficacies and toxicities, manufacturing of the CAR T cells and their clinical application. While a direct comparison of different trial results is challenging due to the complex nature of CAR T cell therapy, factors influencing the design and outcome of CAR T cell trials could be extracted. By the end of 2016, 211 CAR T cell trials were documented, from which 133 target hematologic malignancies and 78 solid tumors. The majority of ongoing trials are conducted in the USA directly followed by China and Europe. There has been a clear focus on CD19-specific CAR T cells for the treatment of B cell malignancies. This might not be surprising given the fact that from 23 published trials assessing CD19-CAR T cells the majority of patients reached either a complete or partial remission as best clinical outcome. When other tumor antigens of the hematologic system or of solid tumors have been selected, clinical benefit was less pronounced. In addition, CAR T cell therapy is often associated with toxicities ranging from being mild to substantial and life-threatening including cytokine release syndrome, neurotoxicity, tumor lysis syndrome, and anaphylaxis as well as on-target/off-tumor recognition. Critical parameters influencing CAR T cell therapy identified are target choice, design of the CAR construct, vector choice, starting material and its handling, preconditioning regimen as well as administration regimen. Overall, CAR therapy needs to be improved with respect to off-target activity, safety and potency. In addition, many obstacles have to be overcome to facilitate the translation of novel CARs into clinics.

Cardiovascular and Pulmonary Diseases

614. siRNA Mediated Silencing of the Epithelial Sodium Channel as a Therapeutic Strategy for Cystic Fibrosis

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Cystic fibrosis is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic AMP-activated chloride channel. CFTR deficiency also regulates the epithelial sodium channel, ENaC, leading to increased sodium and water absorption from the overlaying airway surface liquid. This leads to thickened mucus and impaired mucociliary clearance allowing bacterial infections to become established that contribute to the CF lung pathology.

We are investigating inhibition of ENaC as a therapy for CF by RNA inhibition to restore airway hydration and mucociliary function. Our aims were; 1) to develop and optimise a targeted nanocomplex for penetration of mucus and epithelial siRNA transfection; 2) evaluate molecular and functional effects of ENaC silencing in cell culture models of the human airway epithelium and; 3) assess translational potential of these siRNA nanocomplexes by transfection of normal mice lungs.

The nanocomplexes, called RTNs, comprise non-PEGylated formulations of cationic liposomes and cationic, epithelial receptor-targeting peptides which self assemble on mixing with siRNA. The biophysical properties and transfections efficiency of RTNS were unaltered by nebulisation offering a convenient route for delivery Interestingly the diffusion coefficients of these cationic RTNS and naked siRNA were similar in CF mucus despite their size and charge differences, suggesting that the surface properties of the RTNs facilitate transport through the mucus.

RTNs achieved 30% silencing efficiency for the alpha ENaC subunit after a single transfection of fully differentiated and ciliated primary CF epithelial cells grown in air-liquid interface cultures. This level of silencing of ENaC was shown in Ussing chamber analysis to reduce the short circuit current significantly. We then showed that silencing was accumulative with 50% silencing after three transfections performed at 48 h time intervals We then showed that mucus protein concentration was reduced indicating increased hydration while ciliary beat frequency was increased to normal levels. Thus ENaC silencing of approximately 50% appears to be sufficient to restore parameters of mucociliary clearance in ALI cultured CF cells.

In vivo transfections in normal mice showed that single doses of siRNA delivered by oropharyngeal instillation in mouse lungs silences alpha ENaC by approximately 30% while three doses at 48h intervals resulted

in approximately 60% silencing. In vivo silencing from a single dose persisted for at least 7 days. Treatment was well tolerated with mild levels of inflammation shown histologically.

The siRNA nanoparticles described here are compatible with airway delivery and can overcome the mocociliary barriers to transfection leading to efficient silencing of ENaC in vitro, restoring epithelial hydration and ciliary function. This data supports the hypothesis of ENaC silencing as a therapy for CF and demonstrates a method of in vivo delivery.

615. Regional Venous Infusion of AAV1 or AAV8 Gene Therapy Vectors Results in Robust Muscle Transgene Expression in Rhesus Macaques

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Alpha-one antitrypsin (AAT) deficiency is a genetic disease affecting the lungs due to inadequate anti-protease activity in the pulmonary interstitium. On-going human trials use intra-muscular delivery of adeno-associated virus (rAAV1), allowing expressing myofibers to secrete normal (M)AAT protein. In the Phase IIa trial, patients in the highest dose cohort ($6x10^{12}$vg/kg) were given 100 intra-muscular (IM) injections of undiluted vector, with serum AAT levels still substantially below target levels. Previous work has shown that delivering rAAV vector to the musculature via limb perfusion leads to widespread gene expression in myofibers. We hypothesize that widespread delivery would result in an overall increase in serum AAT levels with the same dose of AAV gene therapy vector and allow for increased volume and thereby dose of vector. In macaques, similar or modestly higher (573.0 ng/ml versus 562.5 ng/nl) serum myc-tagged rhAAT was produced using regional venous infusion when compared to direct IM delivery at the same total vg dose with either rAAV1 or rAAV8, while not being limited to a small volume as with IM injection. These data prove the concept that a 30-fold expanded volume of rAAV-AAT could be delivered to myofibers using limb perfusion without loss of potency on a per vg basis, thereby enabling potential achievement of therapeutic AAT levels in patients.

616. Reprogramming of Adult Mammalian Cardiomyocytes to the Progenitor State via Adenoviral Mediated CCNA2 Expression

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The adult mammalian heart is known to have a very low abundance of progenitor cells, which can take part in active cycling and regeneration after damage. Cardiomyocytes exit the cell cycle soon after birth coincident with the silencing of cyclin A2 (CCNA2). In our previous studies, we demonstrated that viral delivery of *Ccna2* induces cardiac regeneration in infarcted hearts of small and large animal models. We have now optimized culture methods for adult human and mouse cardiomyocytes to investigate the molecular mechanisms of cell division in these cells. The isolated cardiomyocytes start to adhere and spread after 1 week of culture. We induced expression of CCNA2 using adenovirus encoding human CCNA2 cDNA driven by the cardiac specific troponin T (cTnT) promoter. Cytokinesis was visualized using live cell epifluorescence imaging with time lapse microscopy after co-transfecting with adenovirus encoding a GFP reporter driven by cTnT (cTnT-GFP). Approximately 3 fold higher cytokinesis in test samples was observed as compared with controls (cTnT-GFP virus only). We then examined the effect of CCNA2 expression on dedifferentiation of these cells. Isl1 has been shown to be expressed in cardiac precursors and is downregulated upon differentiation. We detected expression of Isl1 in cultured cardiomyocytes transfected with CCNA2-adenovirus while it was absent in null-adenovirus transfected cells. We observed appearance of cardiac progenitor marker "non-muscle myosin IIB" and epithelial to mesenchymal transition markers (Vimentin and FSP1) in these cells along with cardiac marker "troponin Tc". We also examined the gene expression of cardiac markers by qPCR. This was normalized with GAPDH and expression of genes at 3 weeks of culture compared to day 0 and fold ± SEM was determined. We observed decreased expression of adult cardiac markers α-MHC (0.24 ± 0.12), ckmt2 (0.08 ± 0.03) and troponin Tc (0.50 ± 0.02). These observations imply that CCNA2 mediates dedifferentiation of adult cardiomyocytes *in vitro* to a cardiac stem/progenitor cell phenotype which may then re-enter the cell cycle. We are further investigating the potential for these resultant cells to differentiate into functional adult cardiomyocytes *in vitro* and *in vivo*.

617. Molecular Cardiac Surgery Mediated SERCA2a Overexpression Demonstrates Enhanced Cardiac Function and Reduced Borderzone Apoptosis in a 6 Month Ovine Ischemic Cardiomyopathy Model

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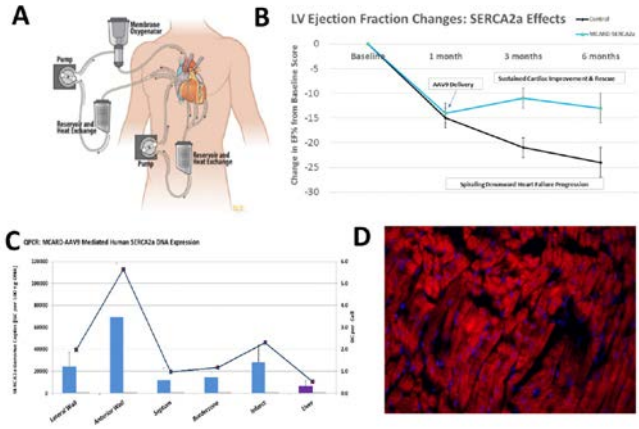
Background The sarcoplasmic endoreticulum ATPase (SERCA2a) is a potent heart failure target having advanced through Phase II trials. Our team has asserted that the recently published trial endpoints were not met due to challenges with the intracoronary infusion route. Our previously validated Molecular Cardiac Surgery mediated delivery method (MCARD) (Fig1A) provides intimate access to the myocardium via complete surgical isolation, leveraging high concentration of vector and favorable AAV perfusion gradients resulting in >1000 fold copy number detection versus standard infusion. As a follow up to previously published AAV1 and AAV9 SERCA2a sets demonstrating rescue at 3 months, we hypothesized MCARD mediated SERCA2a overexpression effects would be retained at 6 months post myocardial infarction with the same robust cardiac specific copy detection. Additionally, emerging data has suggested SERCA2a reduces apoptosis, thus we evaluated in the infarction region.

Methods Twelve sheep were surgically induced with myocardial infarction following baseline MRI and divided between two groups: Control (n=6) and MCARD.SERCA2a (n=6) treated with a single $1x10^{14}$ dose of AAV9.SERCA2a at 1 month. Follow up MRIs were performed at 3 months and at 6 months post therapy. Molecular biology assays for AAV copy genome DNA via QPCR, SERCA2a RNA via RT-QPCR and SERCA2a transduction via confocal stain were performed post mortem normalized to non-treated controls.

Results Cardiac function was in the same range at baseline and 1 month post myocardial infarction. After AAV9.SERCA2a however, the MCARD group vs. Control demonstrated a superior retention of (i.e. change from baseline) ejection fraction at 3 months [-11±2 vs. -21±2%] and at 6 months [-13±3 vs. -24±3%] Fig1B, $p<0.05$. Retaining efficacy at 6 months, MCARD animals also had higher systolic Stroke volume Index [39±2 vs. 31±2 mL/m], improved geometry [1.8±.06 vs. 1.5±.06], and enhanced borderzone contractility [14±4 vs. 5±2] all $p<0.05$ while Controls declined further. Infarction size was the same for both groups. QPCR Fig1C revealed significant genomes (GC) in the heart [69379±48828] vs. off target Liver [6485±4629] GC per 100 ng DNA , $p<0.05$. RT-qPCR detection indicated SERCA2a treated animals had a [60±25] fold mRNA profile vs. Controls [1±.5] $p<0.05$. Myocyte specific SERCA2a protein was found [2±.2] fold overexpressed in the MCARD vs. Control [1±.1] Fig1D $p<0.05$. TUNEL assay revealed [67±6% cells+] in the Control, while MCARD only [39±5%] suggesting a reduction in apoptosis $p<0.05$.

Conclusion MCARD mediated SERCA2a overexpression provides effective and sustainable gene therapy in the presence of significant deterioration post myocardial infarction over the long term. MCARD's unique cardiac specific delivery profile can potentially resurrect SERCA2a gene therapy for various cardiac surgery indications.



618. Efficacy of Plasmid Based Therapy Using Nanoformulations of Anti-Inflammatory Glucocorticoids in Acute Myocardial Infarction

Anthony S. Fargnoli, Michael G. Katz, Charles R. Bridges

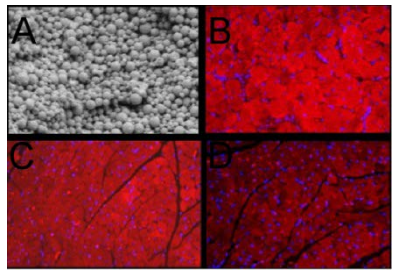
Cardiovascular Research Center, Mount Sinai School of Medicine, New York, NY

Background Improving gene transfer efficiency of DNA plasmids (pDNA) following acute myocardial infarction (MI) is a significant challenge. This process should reduce pDNA susceptibility to blood circulating nucleases, increase myocyte uptake, attenuate immune responses, and improve the cardiac bioavailability of plasmid. Here, we propose to use a nanoparticle formulation (NP) of anti-inflammatory glucocorticoid, solumedrol (SM) to improve plasmid transfection efficiency in the left ventricle after creation of acute MI.

Methods A robust process yielded solumedrol loaded poly lactic glycolic acid nanoparticles, size 150nm, Fig1A. Prior to intramyocardial injection of pDNA, all 12 rats underwent surgically created large infarction and were divided into 3 groups (n=4 ea.) consisting of 2 control groups: intravenous administration of SM (IV/SM),10mg/kg; and intramyocardial (IM) injection of unloaded nanoparticle (IM/NP). The experimental group included intramyocardial injection of nanoparticle formulation of solumedrol (IM/NP/SM), 3mg/kg. All 12 animals then received intramyocardial injection of 300μg of GFP/pDNA in the infarct/border zone. Hearts were harvested at 72 hour timepoint. Histochemistry analysis of infarct/border zone included staining for inflammation, necrosis and apoptosis. Blood cytokines were acquired to measure immunity and SM's effect on inflammation. Confocal analysis of left ventricular tissue selections evaluated the percentage of myocyte GFP expression.

Results All animals survived to 72 hrs and had equal levels of inflammation and apoptosis in the infarct zone. Both the IV/SM [226±65] and IM/NP/SM [227±45] groups had significantly less pro inflammatory TNFα cytokine levels compared with IM/NP group [555±116] in pg/mL, $p<0.05$. The same pattern was confirmed with interleukin-1, with IV/SM [424±121] and IM/NP/SM [367±75] groups. Both groups had significantly less than the IM/NP group [835±147], $p<0.05$. Solumedrol, independent of route, attenuated the host responses in acute MI. Surprisingly however, there was a major difference found in GFP expression in the IM/NP/SM group. Fig1B demonstrating superior percentage of GFP transfected myocytes (red) [71±2%] versus both control IV/SM [57±2%] (Fig1C) and IM/NP [42±4%] groups (Fig1D), $p<0.05$. Interestingly, the IV/SM group did not perform as well as the IM/NP/SM group despite having similar attenuation of cytokines levels.

Conclusion By acting directly in the infarct/border zone, the cardiac specific release of solumedrol nanoparticles can enhance gene transfer efficiency via sustaining a more favorable plasmid trafficking. This concept may improve therapeutic outcomes for gene therapy applications.



619. Isolation of Exosomes from Peripheral Blood Derived Endothelial Progenitor Cells of PAH and Control Subjects with Analysis of Their Surface Markers

Rebecca Harper[1], Michaelia Cockshell[2], Claudine Bonder[2], Paul Reynolds[1]

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Introduction/Aim: Pulmonary arterial hypertension (PAH) is a rare, fatal disease caused by pulmonary vascular remodelling. The disease is characterised by the narrowing of the small pulmonary arteries due to the formation of plexiform lesions and intimal thickening as a result of abnormal endothelial and smooth muscle cell function. These pathological changes lead to the physiological changes of increased pulmonary vascular resistance, ventilation-perfusion mismatching and ultimately respiratory and right heart failure. Despite available therapeutics, survival remains at 54.9% at 3-5 years- thus new therapies are needed. Endothelial Progenitor Cells (EPCs) are important for vascular homeostasis, repair and are known to have a homing ability to the pulmonary vasculature, particularly when trauma or hypoxia is present. Additionally, these cells themselves have altered numbers and function in patients with PAH. Previously, we successfully used 'healthy' EPCs as a therapy to deliver a gene to the pulmonary vasculature in a PAH *in vivo* model. The success of our gene and cell therapy may be due to a paracrine effect, rather than a direct implantation of EPCs into the pulmonary vasculature. We hypothesise that this paracrine effect is due to the release of exosomes (Exos) from the injected 'healthy' EPCs. Thus, EPC derived Exos are an important area of study for understanding the pathogenesis and potential therapeutic approaches for PAH. **Methods:** EPCs were isolated and cultured from both PAH and control patient's peripheral blood (15mL). Following EPC expansion and characterisation, cultures were serum starved for 48 h and the supernatant removed for centrifugal isolation of Exos. These were characterised using western blot and TEM, and assessment of surface marker expression on both Exos and EPCs was conducted via western blot. **Results:** Culture of PAH peripheral blood derived EPCs was developed. A standard protocol to isolate, visualise via TEM and characterise Exo' from both PAH and control EPCs was established. Furthermore, endothelial markers such as CD31, VEGFR2, CD146 and Endoglin were found to have a significantly increased expression on the surface of Exos derived from control EPCs, when compared to EPCs themselves. However, preliminary results indicate there is significantly reduced expression of VEGFR2 on PAH derived Exos compared to control EPCs and Exos, with no difference between the two groups for Endoglin expression. **Conclusions:** We have successfully cultured EPCs from 15mL of PAH patient peripheral blood as well as isolate and characterise Exos from these EPCs. Surface marker analysis of Exos revealed expression of endothelial surface markers with preliminary results indicating differential expression between control and PAH derived Exos. Further analysis of expression differences between control and PAH derived Exos is currently underway.

620. Macrophage-Colony Stimulating Factor Gene Therapy for Murine Hindlimb Ischemia

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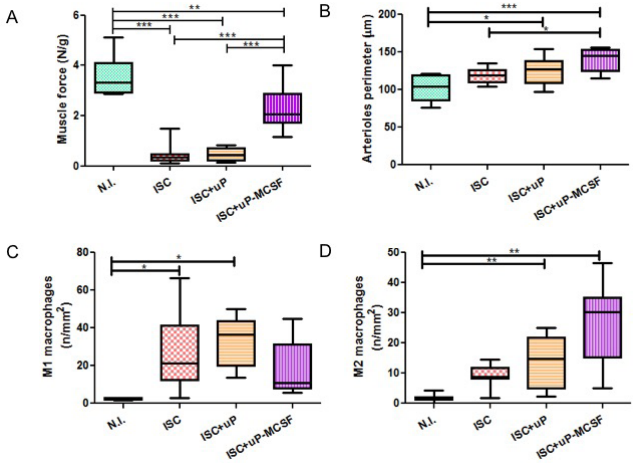
UNIFESP, Sao Paulo, Brazil

Background: Peripheral artery disease is caused by atherosclerosis which narrows or occludes arteries that supply lower limbs, possibly evolving to chronic critical limb ischemia. M-CSF is a hematopoietic factor that promotes proliferation, differentiation and survival of monocytes, macrophages and bone marrow progenitor cells. M-CSF also differentiates preferentially monocytes to M2 macrophages, which are anti-inflammatory and produce angiogenic factors. **Aims:** To assess the effect of M-CSF gene therapy on functional restoration of murine ischemic limbs. **Methods:** Hindlimb ischemia was surgically induced in 10-12 weeks old Balb/c and, three days later, the rectus-femoris muscle was electroporated with 100 μg uP-MCSF plasmid, which expresses constitutively murine M-CSF. During a month after electroporation, M-CSF gene expression in the muscle was quantified by ELISA, blood perfusion was assessed by Laser Doppler Perfusion Imaging (LDPI) system and blood were collected to hemogram weekly. Thirty days after transfection, gastrocnemius muscle force and mass were determined, and the gastrocnemius muscles were analyzed histologically. The following groups were included in this study: Ischemic mice (ISC), ISC electroporated with uP-MCSF vector (I+uP-MCSF), ISC electroporated with uP vector (I+uP) and non-ischemic (N-I) mice. **Results:** M-CSF expression was increased on days 2, 4 and 7, reaching baseline on day 14. The ratio of gastrocnemius force/mass (N/g) was 3.4±0.6 for the N-I group, 0.4±0.1 for the ISC group, 0.4±0.2 for the ISC+uP group and 2.3±1.0 for the ISC+uP-MCSF group, however, LDPI did not show significant difference in blood flow among groups. Hemogram showed a significant increase of monocytes in ISC+uP and ISC+uP-MCSF groups on days 4 and 7 (3.9±0.4 % and 4.4 (day 4); 5.1±0.3 % and 3.6±1.8 % (day 7), respectively). Histological analyses show an increased average vessel perimeter for ISC+uP-MCSF group in comparison to N-I and ISC groups (140.4±15.8; 101.4±17.2 and 118.3±10.4 μm, respectively). **Conclusion:** M-CSF gene therapy increased vessel caliber and improved muscle functionality. **Figure 1** - Functional and histological analyses of gastrocnemius muscle 30 days after gene therapy. A - Isometric tetanic muscle force assessment relative to muscle mass. N/g (newton/gram); B - Arterioles were labeled with α smooth muscle actin by immunohistochemistry and vessels with perimeter between 60 - 300 μm were counted; C - M1 macrophages were stained with anti-iNOS antibody conjugated to peroxidase and macrophages were counted; D - M2 macrophages were stained with anti-arginase I antibody conjugated to peroxidase and macrophages were counted. All analyses were performed using 9 to 10 mice/group. N.I: non-ischemic mice; ISC: ischemic mice; ISC+uP: ischemic mice treated with uP vector; ISC+uP-MCSF: ischemic mice treated with uP-MCSF. Statistical analyses were performed by one-way ANOVA. *$p < 0.05$; **$p < 0.01$ ***$p < 0.001$. uP: empty plasmid vector; uP-MCSF: plasmid vector expressing M-CSF.



621. Combinational AAV Gene Therapy to Treat Multiple Disease States *In Vivo*

Noah Davidsohn[1,2], George Church[3]

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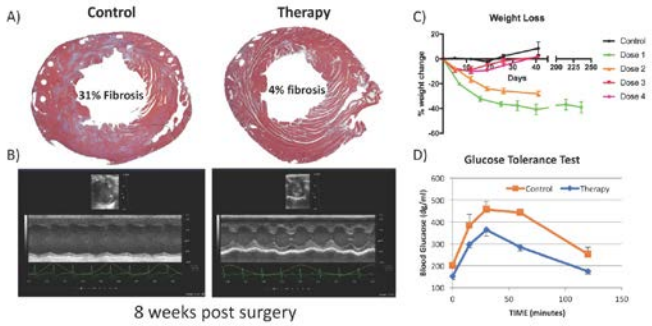
Age is the largest risk factor of developing many diseases including cardiovascular disease, cancer, arthritis, obesity, renal disease, and Alzheimer's disease. There is a complex interplay between various organ and tissue systems that is commonly ignored when treating individual diseases. For instance, one quarter of patients with chronic kidney disease (CKD) develop congestive heart failure (CHF) due to the lack of protective signaling from the kidney. Nevertheless, current healthcare research focuses on treating specific pathologies and disorders individually. Heart failure, obesity and type II diabetes, and renal disease are some of the most common age related diseases in America affecting 45%, 35% and 14% respectively. Many people end up developing numerous comorbidities due to the interconnected nature of all our internal systems. We have developed a single combination gene therapy that can treat three separate diseases, heart failure, obesity and type II diabetes, and renal disease. The aging field has discovered numerous genes that can be modulated for a lifetime with little to no ill effect save for the fact that the mice live longer. We have selected our genes from this subset of safe and overall beneficial genes that had known roles in specific disease processes. Combing these three safe systemic genes we increased the overall well-being of the animal and provide a therapeutic benefit to several disease models as well. The model we used to assess heart failure was a surgical intervention called ascending aortic constriction (AAC). We completely mitigated the effects of AAC surgery with our combinational gene therapy maintaining a sustained fractional shortening (FS) of greater than 30% and decreasing fibrosis up to 85%. We induced obesity and type II diabetes by feeding a high fat diet (HFD) ad lib for greater than 4 months, mimicking the human acquired condition. Through a single injection the mice had sustained weight loss of up to 45% within 20 days post injection. Their glucose tolerance test (GTT) and fasting glucose and insulin had also returned to normal and was slightly better as compared to control mice. To induce renal disease, we used a unilateral ureter obstruction (UUO) model in mice. We alleviated the damage induced by the surgical model established through a decrease in fibrosis and by immuno-histochemical analysis. There have been reports of these three genes showing efficacy in other areas as well (e.g. cognitive dysfunction, cancer, inflammatory diseases, and aging). Our holistic systemic approach to tackling diseases will be more efficacious that inhibiting any one particular pathway. Gene therapy holds the key to permanently and directly modulating the levels of numerous proteins simultaneously to stave off a battery of acquired diseases.



622. Induced Pluripotent Stem Cell Derived Cardiac Myocytes as a Model to Study the Human Desmin R350P Mutation

Kleopatra Rapti[1], Dominik Siede[2], Qinghai Tian[3], Johannes Backs[2], Jochen S. Utikal[4], Peter Lipp[3], Hugo A. Katus[1], Oliver J. Müller[1]

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Desmin is a muscle specific, type III intermediate filament expressed in all muscle cell types: cardiac, skeletal and smooth muscle. Mutations in desmin or its chaperon, αβ crystallin, contribute to desmin-related myopathies, or desminopathies. The desmin p.Arg350Pro missense mutation is the most commonly encountered desmin mutation in Germany and is associated with myopathy, proximal and distal, scapuloperoneal and cardiac myopathy, and cardiac arrhythmia. The underlying mechanism of the disease phenotype caused by this mutation has been studied extensively both in *in vivo* mouse models and in *in silico* models. Nonetheless, this mutation results in a wide range of clinical phenotypes in humans. It is therefore vitally important to study this mutation in a patient relevant or specific background. Induced pluripotent stem cells (iPSCs) are derived from individual patients and they are extensively used to model human disease in the past decade. There are well-established methodologies to differentiate them to cardiac myocytes (CMs), which provide valuable insights into the disease and offers the potential to cell replacement therapies. Based on the potential of this technology, we report the generation of iPSC from one patient carrying this mutation and the differentiation to CMs. The iPSC-CMs show expression of cardiac markers and during their differentiation they show the expected temporal expression of genes involved in mesoderm, precardiac mesoderm, precardiac and

cardiac development. In preliminary studies, we are investigating the expression and structural profile of several cardiac markers in mutant (desmin R350P) and wild-type hiPSC-CM. We also investigate several other desmin mutations based on the wild-type hiPSC-CMs and finally the suitability of this *in vitro* system for studying novel gene therapy approaches.

623. High Efficiency *In Vitro* Transduction of Human Airway Basal Cells

Nigel Farrow[1,2], Martin Donnelley[1,2], Nathan Rout-Pitt[1,2], Chantelle McIntyre[1,2], Ivan Bertoncello[3], David W. D. Parsons[1,2]

[1]Respiratory and Sleep Medicine, Women's and Children's Hospital, North Adelaide, Australia, [2]Robinson Research Institute, and Adelaide Medical School, University of Adelaide, Adelaide, Australia, [3]Lung Health Research Centre, Department of Pharmacology and Therapeutics, University of Melbourne, Melbourne, Australia

Introduction: To develop an airway gene therapy for treating CF airway disease the transduction of resident airway stem or progenitor cells has clear potential. The successful gene correction of basal cells should allow for the correction to be passed on to the terminally-differentiated airway cells that arise from these corrected epithelial stem cells, including ciliated cells that are a key target for CF airway gene therapy, ensuring benefit is sustained. Here we tested if human airway basal cells in culture could be effectively transduced with a human-based (HIV-1) lentiviral vector.

Methods: Normal Human Primary Bronchial Epithelial Cells obtained from LONZA (CC-2540S) were seeded, expanded and passaged (as per manufacturer's instructions) in Bronchial Epithelial Growth Medium (BEGM, CC3170). Once 90% confluent at passage 2 the cells were harvested and seeded into 6 well plates at 2.5 x 10^5 cells per well (~70% confluence). Untreated control cells were used for immunohistochemistry staining against keratin 5 to confirm basal cell identity. Our lentiviral gene vector (HIV-1 based, VSV-G pseudotype, driven by the EF1α promoter) containing the LacZ reporter gene (1.4 x 10^9TU/ml) was applied to cells 2 hours after seeding at MOI's of 100, 10 and 1, (n=3 per group) and left overnight. Standard X-gal processing after 3 days revealed the level of LacZ expression. **Results**: Anti-keratin 5 staining showed that the cells at passage 2 were predominantly human airway basal cells. Human airway basal cells showed essentially complete LacZ transduction at an MOI 100: 99.9% (0.3% (SD)) and at an MOI of 10: 97.7% (0.7%); at an MOI of 1 transduction was 47.4% (3.7%) of the cells. **Conclusion**: This study validates that human airway basal cell cultures can be efficiently transduced with our LV gene vector, and may be indicative of the likely *in vivo* efficiency. High levels of basal cell transduction are important for ensuring long-lasting therapeutic effect, and to enable use of lower vector doses to potentially reduce inflammatory and immune function side effects. In addition, high efficiency transduction could also be useful for alteration of gene expression in airway basal stem cells destined for *ex vivo* manipulation for cell therapy purposes. **Acknowledgements**: Supported by USA CF Foundation, with supplementary support from the Cure4CF Foundation SA.

Cell Therapies III (T-Cell Immunotherapy)

624. CAR T-Cells with Induced Secretion of Helicobacter Pylori Neutrophil-Activating Protein (HP-NAP) Yields Improved Anti-Tumor Activity and Reduced Immunosuppression

Magnus Essand, Jing Ma, Chuan Jin, Mohanraj Ramachandran, Di Yu

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Chimeric antigen receptor (CAR)-engineered T-cells, targeting the pan B-cell marker CD19, have made remarkable progress in the clinics for both acute and chronic B-cell leukemias and to some extent also for B-cell lymphomas. However, the semi-solid structure of lymphomas appears to impede infiltration of CAR T-cells and the immunosuppressive tumor microenvironment dampens CAR T-cell activity. These obstacles are even more pronounced for solid tumors. Another problem for CAR T-cell therapy of solid tumors is target antigen heterogeneity within tumors and toxicity related to the fact that target antigens are also expressed on normal cell types. Therefore, CAR T-cells targeting solid tumors must be able to induce bystander activity to kill also antigen-negative tumor cells and at the same time protect healthy tissues. In order to improve CAR T-cell therapy for lymphomas and solid tumors, we have engineered CAR T-cells with a transgene encoding *Helicobacter Pylori* Neutrophil-Activating Protein (NAP) that is expressed and secreted upon CAR T-cell recognition of antigen-positive tumor cells. We have previously demonstrated strong immune activating properties of HP-NAP. Herein, we evaluate both CD19-CAR T-cells and GD2-CAR T-cells with inducible secretion of HP-NAP and we found that NAP expressed by activated CAR T-cells matured autologous DCs *in vitro* and stimulated them to secret key cytokines (IL-12, IL-1α/β) and chemokines (CXCL11, CXCL12) for T helper type 1 (Th-1) induction. The secreted HP-NAP was also chemoattractant for neutrophils, monocytes and NK-cells. When NAP-CAR T-cells were co-cultured with immature DCs and antigen-positive target cells, HP-NAP secreted upon target cell recognition was able to mature the DCs, leading to a reciprocal effect on the CAR T-cells with improved *in vitro* cytotoxicity. Systemic treatment with HP-NAP-secreting CAR T-cells reduced syngeneic tumor growth in fully immune competent mouse models, recruited neutrophils to the tumor bed and reduced the amount of tumor-associated monocytic myeloid-derived suppressor cells. HP-NAP secretion may therefore become a useful component for development of CAR T-cell therapy for solid tumors.

625. *In Vitro* Characterization of CD19-Directed CAR T-Cell Preparations Reveals Phenotypic Divergence and Reduced Functional Activity of Cynomolgus Monkey versus Human CAR T-Cells

Jennifer Marlowe[1], Linda Dong[2], Kellie Latimer[1], Xiaorui Yao[2], Laura Beasley-Topliffe[1], Natalie Claudio[1], Alberto Del Rio Espinola[3], Joseph Gabriels[1], Daher Ibrahim Aibo[2], Valentina Jeliazkova[1], Ned Kirkpatrick[1], Tim MacLachlan[1], Maria Magnifico[1], Keith Mansfield[1], Lori Martin[2], Jonathan Moggs[3], Jill Omerza[2], Andrea Pomerantz[1], Farid SariSarraf[1], LeeAnne Talbot[2], Sarah Voytek[1]

[1]Novartis Institutes for Biomedical Research, Cambridge, MA, [2]Novartis Institutes for Biomedical Research, East Hanover, NJ, [3]Novartis Institutes for Biomedical Research, Basel, Switzerland

T cells modified to express chimeric antigen receptors (CAR) directed towards specific tumor cell surface antigens have proven to be powerful treatments for a growing number of cancer types, particularly hematological malignancies. There is currently a lack of relevant nonclinical animal models for the assessment of potential safety concerns that may arise as the result of cell surface antigens expressed in normal tissues, as is the case for many solid tumor antigens. We generated CD19-targeting CAR T-cells from four cynomolgus monkey donors for the purpose of assessing their *in vivo* and *in vitro* functionality. One out of four donor T cells exhibited the ability to deplete engrafted CD19-expressing NALM6 cells in an NSG mouse tumor model, concomitant with peripheral CAR T-cell expansion. T cell doses of 50 to 200x10^6/kg into each of the four cynomolgus monkey donors resulted in no discernable peripheral expansion of the CAR+ T-cell population, no detectable cytokine increases, and no depletion of CD19-expressing B cells. In order to further investigate this lack of response to CAR T-cells in the cynomolgus monkey, we conducted a series of phenotypic and *in vitro* functional characterization assays on human and cynomolgus monkey CAR T-cell preparations expressing an identical CD19-directed CAR construct and prepared using identical transduction and analogous cell expansion protocols, and tested against K562 cells expressing either human or monkey CD19 target. These assays indicated that the human CAR T-cell preparations were more effective at target cell killing and T cell activation than cynomolgus monkey CAR T-cells. In addition, human CAR T-cells released significant levels of IFNγ and IL-2 in response to target antigen, whereas monkey CAR T cells did not, and this response was more pronounced in the presence of human target relative to monkey target. Imaging flow cytometry experiments also indicated that human CAR T-cells showed a more robust localization of the CAR to the immune synapse when exposed to target cells. Immunophenotyping of human and monkey CAR T-cell preparations indicated that monkey cells are predominantly CD8-positive and express high levels of PD-1, PD-L1, and CD69, and are largely HLA-DR negative, suggesting potential mechanisms for the relative lack of activity of the monkey cell preparations as compared to human CAR T-cell preparations. Overall, our results suggest that the cynomolgus monkey may be a poor model for recapitulating the pharmacological effects of CD19-directed CAR T-cells observed clinically, potentially owing to multiple phenotypic differences and functional deficiencies relative to human CAR T-cell preparations.

626. Mouse CD19-Targeted CAR T Cells with a Humanized 41BB Domain Improve Leukemia Killing and Memory Function *In Vivo*

Gongbo Li[1], Nolan Beatty[2], Paresh Vishwasrao[1], Bin Yu[1], Justin Boucher[1], Yongliang Zhang[1], Marco Davila[1]

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CD19-targeted CAR T cells carrying a co-stimulatory domain, either CD28 or 41BB, have generated excitement due to clinical success treating CD19+ malignancies, especially B-ALL (B cell acute lymphoblastic leukemia) and DLBCL (Diffuse Large B cell lymphoma). Despite similar CR (complete response) rates and survival in clinical trials, recent studies have suggested that CAR T cells with CD28 or 41BB co-stimulatory domains function differently. Studies of human CD19-targeted CAR T cells suggest that CD28 CAR drives an effector/effector memory phenotype, while 41BB CAR drives a central memory phenotype. However, these studies have largely relied on *in vitro* or gene expression studies and are limited by confirmation of these functional attributes in relevant animal models. Therefore, we created fully murine CD19-targeted CARs with either CD28 or 41BB to evaluate in a B-ALL immune competent mouse model. Mouse 41BB CAR was found to be surprisingly inferior to CD28 CAR based on survival and *in vivo* persistence. However, this inferior function could be rescued by replacing mouse 41BB with its human counterpart. We determined that the hum41BB co-stimulatory domain in an otherwise fully murine CD19-targeted CAR imparted protection against B cell leukemia at a level similar to mouse CD28 containing CAR T cells (Figure 1).

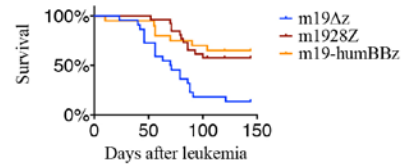

Figure 1. Survival of Eμ-ALL injected B6 mice after treatment with cyclophosphamide and 3e5 CAR T cells.

We then evaluated the persistence of CD28 and hum41BB containing CAR T cells in mice that lack CD19 antigen. Both CD28 and hum41BB CAR T cells persisted in an antigen-independent manner (Figure 2) and proliferated upon early antigen challenge.

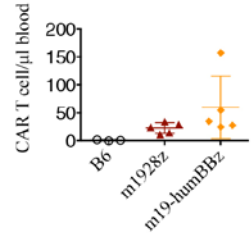

Figure 2. Antigen-independent persistence of CD19-targeted CAR T cells in the blood of CD19-deficient mice 8 weeks after infusion.

Furthermore, both CD28 and hum41BB CAR T cells remained antigen-sensitive 2 months after adoptive transfer based on the release of cytokines upon encountering target cells. However, only hum41BB CAR T cells were able to proliferate after a late antigen challenge, demonstrating a functional hallmark of memory T cell function. Further immune phenotyping suggested that the difference in long-term antigen sensitivity correlated with expression of exhaustion markers. Our study sheds light on how CD28 and 41BB CAR T cells function differently in animal models and also suggests methods for potential improvement of these therapies in patients.

627. BAFF Receptor (BAFF-R)-Redirected T Cells Efficiently Target B-Cell Acute Lymphoblastic Leukemia (B-ALL)

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B-cell Acute Lymphoblastic Leukemia (B-ALL) is most common in children (80%), but it has also a peak of incidence in adult age. Recently, immunotherapeutic approaches targeting the CD19 molecule have demonstrated remarkable success in the treatment of relapsed and refractory B-ALL, which remains a major clinical need. Important downsides of these strategies are the emergence of CD19-negative relapses and B-cell aplasia as a result of anti-CD19 CAR T-cell persistence. In this context, we hypothesized that the receptor for B-cell activating factor (BAFF-R), a transmembrane protein fundamental in B-cell maturation and survival, could be an interesting molecule to be targeted, taking the advantage that this receptor is undetectable on bone marrow B-cell precursors. Here we showed that BAFF-R is highly expressed in B-ALL primary samples at the onset and relapse. In order to develop a chimeric antigen receptor (CAR) approach targeting BAFF-R molecule, six anti-BAFFR CAR genes that differ for the inversion of the VH and VL and the length of the spacer domain have been generated. Cytokine-induced Killer (CIK) cells, engineered using an improved *Sleeping Beauty* (SB) transposon system, stably expressed anti-BAFFR.CARs, and maintained their characteristic phenotype. Among the newly constructed CARs, the shortest VHVL CAR exerted the highest anti-leukemic activity towards target cells, such as NALM-6, with an *in vitro* killing activity of 60%. We also evaluated later effector functions in terms of cytokine release by intracellular staining (8,9±2% of IFN-γ and 16,4±5,5% of IL-2 producing cells). Importantly, we also detected a specific cytotoxic activity towards primary B-ALL blasts (average 65,6±4,5%, n=9). Combining the INVsh.CAR with CD19. CAR we detected a superior antitumor activity towards ALL targets. Furthermore, by using a sample collected from a patient relapsed with CD19 negative disease, we demonstrated the ability of the INVsh. CAR to lysate CD19-negative blasts. Taken together, these findings make this receptor a safe and attractive target for a second line B-ALL immunotherapy in case of relapse after CD19-targeting therapies or for a double targeted approach. Being restricted to mature B cells, but absent on precursors and plasmablasts, our strategy could have an inferior toxicity concerning the emergence of B-cell aplasia observed in patients treated with anti-CD19 CAR-modified T cells.

628. Clinical Scale Production of Leukemia Specific T-Cells from Non-Transplantable Cord Blood Units as a Paradigm of Circular Economy

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Introduction. Immunotherapy with leukemia-specific T cells (leuk-STs) is a promising strategy for leukemia patients relapsing after hematopoietic stem cell transplantation (HSCT). The need however, for high numbers of antigen presenting cells (APCs) to generate clinically relevant doses of leuk-STs, represents a major challenge. Low volume, non-transplantable cord blood units could potentially serve as a rich and off-the-shelf, indirect source of dendritic cells (DCs) for the production of leuk-STs with reduced alloreactivity. We here aimed to generate leuk-STs targeting the leukemia-related antigens, Wilms tumor protein (WT1) and Preferentially Expressed Antigen in Melanoma (PRAME), by exploiting low volume, non-transplantable CBUs. **Materials and Methods.**To generate DCs, $CD34^+$ cells immunomagnetically enriched from non-transplantable CBUs (≤70ml), were cultured in G-rex devices in the presence of SCF, GM-CSF and IL-4. Maturation of DCs was induced by Toll-Like Receptor ligand 3 (polyI:C) and 7/8 (R848). Matured DCs were immunophenotypically characterized by flow cytometry (FCM) and secreted cytokines were measured with ELISA. Matured DCs were activated with a peptide-mix of WT1+PRAME and used subsequently as APCs to repeatedly stimulate naïve T-cells (derived from the $CD34^-$ fraction of the same CBU). The phenotype and specificity of generated leuk-STs were determined by FCM and IFN-γ/ELISpot, respectively. **Results.**By culturing $4.2x10^5$±$1.1x10^5$ $CD34^+$ cells from 4 non-usable CBUs, we generated a median of $3.3x10^9$($1.9\text{-}5.7x10^9$) myeloid DCs ($CD33^+$/$CD11c^+$:76.8±5.5%) in 35 days (fold change~11.000). The produced cells expressed maturation markers ($CD40^+$:79±12%, HLA-DR^+:78±10%) and secreted high levels of Th1-cytokines (IL-12:224±185pg/ml, IL-6:19±$1x10^4$pg/ml, TNF-α:5268±1316pg/ml) and low levels of the Th2-cytokine, IL-10. At 4 weeks, expanded cells reached clinically relevant doses of a mean $7.5±3.4x10^7$ CD3+ cells. Generated cells were polyclonal, expressed effector memory ($CD45RA^-$/$CD62L^-$:52.8±5%)/effector memory RA markers (TEMRA: $CD45RA^+$/$CD62L^-$:46±4%)and contained insignificant amounts of $CD4^+$/$CD25^+$ cells (1±0.5%). Specificity was obtained after the 2nd stimulation at the earliest, and was increasing after each stimulation [mean spot forming cells (SFC)/$2x10^5$ cells at 2nd,3rd,4th stimulation: 106±33; 422±111; 1335±314; respectively]. At the end of the culture, produced cells were highly specific for both targeted antigens (PRAME:1019±275, WT1:316±55), while Programmed cell death protein-1 expression was not significantly increased ($CD3^+$/PD-1^+:9±4%), implicating absence of cell exhaustion after repeated stimulations. **Conclusions.** We here report a paradigm of "circular economy" in science through the exploitation of CBUs disqualified for transplantation, towards the clinical scale generation of leuk-STs

and the future establishment of third-party leuk-STs banks. Whether similarly produced leuk-STs could significantly advance the treatment of leukemic relapse after HSCT, it will be ultimately determined by *in vivo* preclinical and clinical studies.

629. PiggyBac Mediated T Cells Expressing Anti CD19 Chimeric Antigen Receptor for a Clinical Trial

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[Introduction] Adoptive transfer of T cells expressing anti CD19-chimeric antigen receptor (CAR) has clinically succeeded in CD19-positive hematological malignancies. We previously showed that the *piggyBac* transposon system is a promising genetic tool for stable, non-viral gene engineering of primary human T cells. We have now improved these process and construct to avoid magnetic cell isolation, shorten the culture period, and increase *in vivo* efficacy to provide clinical grade CD19.CAR T cells. [Methods] CD19.CAR-T cells were produced from peripheral blood mononuclear cells (PBMC) of healthy donors by electroporation using two vectors encoding CD19. CAR and *piggyBac* transposase, respectively. All cells were cultured in serum-free medium (TexMACS) containing IL-7 and IL-15 in 24-well plates. Electroporated cells were immediately transferred to irradiated autologous activated T-cells (ATCs) pulsed with 4 viral peptide pools (PepTivator; AdV5 Hexon, CMV pp65, EBV EBNA-1, and BZLF1). All cells were transferred into G-Rex10 culture device with peptide-pulsed irradiated ATCs on day 7 and cultured until day 14. The phenotypes of CAR-T cells were determined by flow cytometry. The anti-leukemic function was examined by in vitro coculture assay and in vivo xenograft tumor model. The integration sites of CAR genes were detected by inverse polymerase chain reaction and subsequent next-generation sequencing using MiSeq. Proto-oncogenes were defined according to the Cancer Gene Census of the Catalogue of Somatic Mutations in Cancer database. [Results] The median number and transduction efficiency of CAR-T cells at day 14 obtained from $2x10^7$ PBMC in 9 donors were $1.0x10^8$ (range, $0.58\text{-}1.8x10^8$) and 51% (range, 29-73%), respectively. The major subset of CAR-T cells was phenotypically CD8+CD45RA+CCR7+, closely related T-memory stem cells. In 7 days coculture assay, CD19.CAR-T cells mostly eliminated CD19 positive tumor cell lines at an E:T ratio of 1:5. In NSG mice inoculated with CD19 positive tumor cells, CD19.CAR-T cells dramatically inhibit tumor growth. High throughput sequencing to examine the *CAR* integration pattern revealed the frequency of integration within 50 kb of transcription starting site of proto-oncogene of *piggybac* was comparable with that of retrovirus (3.9% vs 5.1%, p=0.163) while less than that of lentivirus (3.9% vs 5.7%, p=0.0219). *PiggyBac* had no integration into or near mutagenesis genes reported in HSC-based gene therapy, such as *LMO2*, *BMI1*, *CCND2*, *MDS1*, *MN1*, *MDS1/EVI1*. [Conclusion] These preclinical data support a *piggyBac* transposon technology as cost-effective and safe therapeutic platform for CD19. CAR-T cell therapy for the clinical trial.

630. Design and Development of a Chlortoxin-Targeted Chimeric Antigen Receptor for Treatment of High-Grade Brain Tumors

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High-grade gliomas are the most aggressive and refractory brain tumors. The conventional therapies are generally lack specificity and cannot address highly invasive and infiltrative tumor nature. In the scope of rapidly developing targeted interventions, adoptive transfer of immune cells genetically modified with Chimeric Antigen Receptors (CARs) was applied with uneven success. Several CARs targeting brain tumor antigens (IL13Rα2, HER2/ERBB2 and EGFRvIII) have been tested in preclinical settings, with transient response seen in two phase 1 clinical trials targeting IL13Rα2. Clearly, a successful clinical translation of targeted cell-based therapies will greatly benefit from an exploration of new glioma targets, optimization of the CAR design, as well as immune effector cell type selected for CAR grafting. We have designed a novel CAR based on Chlorotoxin (CTX) peptide as a targeting moiety of the CAR molecule (CTXCAR). Chlortoxin is a short natural peptide isolated from the venom of the Israeli Yellow Scorpion. It efficiently binds to GBM cells in a grade-related manner with no measurable cross reactivity to normal brain. These properties have resulted in studies that investigated the use of the CTX peptide for tumor imaging and targeted delivery of synthetic peptide in its free form or conjugated with low molecular tags. However attempts to test CTX in the context of complex molecules, such as CARs, to our knowledge have not been attempted. We first designed and evaluated CXTCAR properties using 3rd generation CAR scaffold, which includes the intracellular domains of CD28, OX40, and zeta chain of the T-cell-receptor. The expression of CTXCAR and control CAR (having irrelevant short peptide instead of CTX, noCTXCAR) was tested in different effector cells upon retroviral transduction. We demonstrated efficient transduction of CTXCAR in Jurkat (43-90% transduced cell), αβT cells (25%-67%) and γδT cells (20%-62%). Cells expressing CTXCAR preferentially bind to glioma cell lines in cell-cell binding assay compared to cells expressing noCTXCAR (14% versus 6% in U87 cells and 20% versus 10% in U251 cells). CTXCAR-expressing cells also more efficiently bind recombinant MMP2, one of the main known molecular targets of CTX. Finally, αβT cells expressing CTXCAR demonstrated higher cytotoxicity towards target glioma cell lines. In summary, CTX can be used as targeting moiety in the context of glioma-targeted CARs and its potential efficacy and safety need to be tested further in preclinical models.

631. Regeneration of T Helper-Like Cells That Induces Cytotoxic T Cell-Mediated Antitumor Immunity

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The activation of $CD4^+$ T helper (Th) cells is crucial for the induction of cytotoxic T lymphocyte (CTL) responses against malignancy. We reprogrammed a Th clone specific for chronic myelogenous leukemia (CML)-derived b3a2 peptide to pluripotency and re-differentiated the cells into T-lineage cells (iPS-T cells). Although iPS-T cells retained the same T-cell receptor (TCR) as the original Th clone, their gene expression patterns resembled those of group 1 innate lymphoid cells, and CD4 molecule, an essential co-receptor in TCR-mediated Th responses, was not expressed. The transduction of CD4 genes into iPS-T cells enhanced b3a2 peptide-specific responses. iPS-T cells contained a subpopulation that up-regulates CD40 ligand (CD40L) in response to IL-2 and IL-15. Besides CD4 expression, $CD40L^{high}$ iPS-T cells induced antigen-specific DC maturation characterized by enhanced CD83, CD86, and CCR7. In the presence of Wilms tumor 1 (WT1) peptide, DCs conditioned by CD4-modified $CD40L^{high}$ iPS-T cells stimulated the priming of WT1-specific CTLs. These CTLs eliminated WT1 peptide-expressing CML cell lines *in vitro* and *in vivo*. Our findings indicate CD4 modification and purification of $CD40L^{high}$ iPS-T cells generates T helper-like cells that induce effective anti-leukemic CTL responses via DC maturation and suggest feasibility of the cells for adoptive immunotherapy against leukemia.

632. A Flow Cytometry Based Evaluation of T Cells Mediated Cytotoxicity to Monitor Safety and Efficacy in Clinical Immunotherapy Trials

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Background: Cell-mediated cytotoxicity is typically assessed by measuring the release of radioactive chromium (Cr-51) from labeled target cells. Despite low sensitivity, this remains the gold standard assay for evaluation of lymphocyte-mediated allogeneic cell killing and several clinical protocols use Cr-51 to evaluate for alloreactivity in T-cell therapeutics. A flow-cytometry based assay may yield greater sensitivity in the detection of cytotoxic T cell activity, while eliminating the need for radioactivity. **Objective**: To evaluate the performance of a flow-cytometry based cytotoxicity assay that measures the cleavage of caspase-3 (casp-3) against the standard Cr-51 release assay. **Methods**: 12 donor-derived cytotoxic T lymphocyte (CTL) cell lines were used in parallel assays of allogeneic cell killing. In test assays, CTLs were incubated together with allogeneic dye-labeled target cells for 4 hours at effector:target cell ratios of 5:1, 10:1, 20:1, and 40:1. Cells were then permeabilized and incubated with a monoclonal antibody specific for cleaved casp-3. Cleaved casp-3 levels in dye-labeled target cells were determined by flow cytometry. In control assays, the CTL lines were evaluated using the conventional Cr-51 release assay. Results of test and control assays were comparatively evaluated in terms of % cell killing. **Results**: At the clinically relevant 20:1 effector:target cell ratio the mean cell killing as determined by cleaved casp-3 was -0.99%±4.5%, versus -2.5%±3.2% by conventional Cr-51 release. The two methods were not found to differ significantly by paired t-test (p=0.34). Agreement between the two methods was also determined by Bland-Altman analysis, which revealed a small ratio (Cr-51/Casp3) versus mean (bias= -0.45, SD 3.6) suggesting that the two methods tend to be equivalent in detection of alloreactivity. All the CTL products evaluated were released for clinical use and infused to patients without causing Graft-versus-Host Disease. Further, we demonstrated that the casp-3 cleavage assay could measure specific CTL mediated killing (at 20:1 ratio) of target cells pulsed with HIV, EBV, and CMV viral peptides; yielding values of 80.9%, 46.8%, and 10.8% cell killing, respectively. **Conclusions**: To our knowledge this is the first time that a flow cytometry based evaluation of CTL mediated cytotoxicity has been compared to the standard Cr-51 assay as a means to monitor safety and efficacy in clinical immunotherapy trials. Our observations suggest that the casp-3 cleavage assay compares favorably to the Cr-51 release assay and warrants further testing as a means of reliably and reproducibly detecting alloreactivity and antigen-specific cytotoxicity of T cell products manufactured for clinical use.

633. The Future of T-Cell Therapy - Fully Closed and Automated Manufacturing Using Novel Capture Particles

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T-cell therapies have demonstrated clinical efficacy and are now on the verge of commercial production. Current clinical manufacture utilizes open, manual processing in a controlled environment room, often requiring multiple platforms, operated by highly skilled technicians. Although feasible at a low volume, there is a requirement to expand the manufacturing capability to meet commercial product demand whilst ensuring consistent quality, scalability, sustainability and a reasonable cost of goods. The data presented in this study demonstrates the ability to perform all pre-culture (debulking, cell isolation, culture inoculum formulation) and post-culture steps (harvest, wash, volume reduction, final product formulation) in a completely closed and automated manufacturing platform utilizing counter-flow centrifugation (CFC platform). The programmable CFC platform allows multiple unit operations to be conducted on one platform, reducing the manual transfer stages, multiple disposable sets and reducing the capital expenditure required for multiple platforms. Completely closing the process reduces the contamination risk and allows a lower classification cleanroom permitting reduced facility costs. This also allows for multiple patient lots to be manufactured in the same room, with the same operator oversight, heavily reducing the labor time and therefore allowing a sustainable, affordable scale out approach, necessary for patient-specific manufacturing. Figure 1 depicts the process flow moving from fresh apheresis through to an isolated, cryopreserved

T-cell product. After efficiently removing platelets, the targeted cells, in this case T cells, are bound to biotinylated target-specific antibodies which in turn are bound to streptavidin MagCloudz beads from Quad Technologies. The labeled T cells are then isolated using the size and density properties of the MagCloudz. MagCloudz have the unique properties of a phase change hydrogel that following isolation allows the MagCloudz capture particle to be detached and separated from the target T-cells. This novel method of enrichment allows the target cells to continue in the manufacturing process free from any bound capture particles, which contrasts with the current standard. The T-cells are then formulated into culture medium at the required concentration for sterile weld and direct transfer from the CFC platform into a closed, controlled culture system. Following culture, the system is then sterile welded to the CFC platform, the cells captured in a fluidized bed, washed, and formulated into cryo-solution and transferred into a final container for cryopreservation. This work demonstrates the ability to manufacture T-cells incorporating a completely closed, automated platform, coupled with a novel isolation technology, MagCloudz. This has the potential to transform the current standard of T-cell manufacture and make these therapies accessible to all.

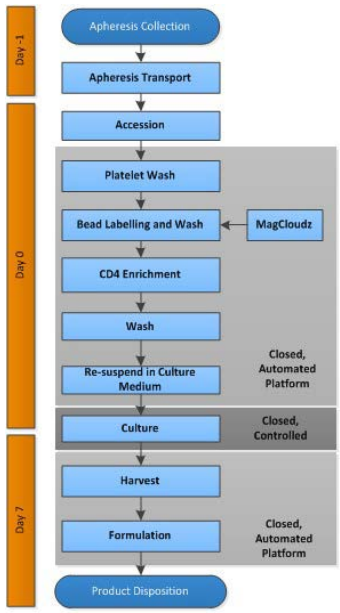

Figure 1: Process flow Diagram - Automated t-cell manufacturing process.

634. 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 auto-immune diseases. One limitation of current adoptive T-cell therapeutics is a lack of control over cell proliferation following engraftment-patient toxicities have been linked to unregulated cell expansion, and lack of potency with lack of cell persistence. Here, we evaluated a panel of synthetic drug-dimerizable cytokine receptors to identify receptor architecture(s) capable of driving proliferation of primary human T cells. One architecture, which we have termed chemically-induced signal complexes (CISCs) showed the capacity to drive drug-dependent human T-cell expansion *in vitro* without exogenous cytokine supplementation. Flow analyses demonstrated the capacity of the drug-induced CISC signaling to selectively expanding CISC-expressing cells within a mixed population. Applications of the CISC architecture with alternative dimerization approaches, gene editing, and for *in vivo* T-cell regulation will be presented. The CISC architecture establishes a new technology for regulated cell expansion which may be broadly applicable to adoptive cell therapies.

153. Magnetic Guidance for Accelerating Endothelium Recovery in Injured Arteries

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Increased susceptibility to thrombosis, neoatherosclerosis and restenosis due to incomplete regrowth of the protective endothelial layer remains a critical limitation of the interventional strategies currently used clinically to relieve atherosclerotic obstruction. Rapid endothelium recovery holds promise for both preventing the thrombotic events and reducing post-angioplasty restenosis, providing the rationale for developing new approaches for accelerating arterial reendothelialization. In the present study, we applied a two-source magnetic guidance scheme to achieve stable homing and site-specific expansion of endothelial cells (EC) in injured arteries implanted with stents. EC functionalized with biodegradable magnetic nanoparticles (MNP) exhibited unaltered proliferation rates, capacity for cryopreservation with fully retained cell viability, as well as strong magnetic responsiveness enabling magnetically controlled, targeted delivery to stented arteries *in vivo*. Using bioluminescent imaging and fluorimetric tissue analysis, we showed that 1) magnetically guided cells will home and expand at the site of stent implantation in a rat model of stent angioplasty, 2) targeted cell delivery realized with a brief exposure to a uniform magnetizing field of a clinically applicable strength can markedly improve site specificity as evidenced by the target:nontarget ratio dramatically increased in comparison to non-magnetically treated control animals, and 3) elimination of MNP used for EC functionalization will proceed concomitantly with local cell expansion after the completion of the targeted delivery step. The present study contributes to establishing effectiveness and safety of magnetically guided cell delivery and shows the potential of this approach for accelerating the healing of injured blood vessels and reducing vulnerability to untoward effects associated with stent angioplasty.

Gene Targeting and Gene Correction III

635. Successful Editing of the *CD40LG* Locus in Human Hematopoietic Stem Cells

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X-Linked Hyper-IgM Syndrome (X-HIGM) is a primary immunodeficiency resulting from mutations in the gene encoding CD40 ligand (*CD40LG).* CD40L is expressed on the surface of activated T cells, and has a crucial role in follicular T cell help, including signaling isotype switching in B cells during an infection. Affected individuals experience recurrent infections, neutropenia, autoimmunity, and have an increased risk of malignancies. Intravenous immunoglobulin or allogeneic bone marrow transplant are the standard treatments; however, these have attendant impacts on the quality of life, and many patients lack a suitable donor. Genetic therapies could provide an important treatment option for this disease. Notably, previous studies of gene replacement therapy in mice demonstrated that vectors that did not mediate an endogenous pattern of CD40L expression resulted in lymphoproliferation and severe toxicity. We thus previously devised a strategy based on gene editing to integrate a CD40L cDNA downstream of the endogenous *CD40LG* promoter in primary human T cells. Using *CD40LG* TALENS and an AAV donor template for homology directed repair, we reconstituted endogenous-like CD40L expression patterns on edited T cells and rescued functional activity in X-HIGM patient cells. In order to develop a stable X-HIGM treatment, we report here the application of our gene editing strategy at *CD40LG* in adult human $CD34^+$ peripheral blood stem cells. TALEN mRNA or CRISPR/Cas9 RNPs targeting exon 1 were combined with delivery of an AAV6 donor template containing an MND promoter-GFP expression cassette flanked by 1 kb *CD40LG* homology arms. This donor template allowed us to track editing rates within the *CD40LG* locus (normally silent in hematopoietic stem cells) by flow cytometry. We achieved editing rates of ~25% with TALEN and ~35% with CRISPR nuclease platforms. Importantly, our editing strategy minimally impacted viability and differentiation potential as measured *in vitro* by colony forming assays. This was validated *in vivo* in cells that were engrafted into NSG mice, where we found minimal reduction in human CD34+ engraftment of edited cell populations compared to mock edited populations. Of note, ratios of myeloid, B and T cells are similar between GFP+, GFP- and non-edited control hCD34 cells. Current work focuses on identifying key conditions that facilitate gene editing and engraftment of long term HSCs with the goal of developing a platform for translation of this approach into clinical application.

636. CRISPR/CAS9-Mediated Monoallelic Knockout of the CXCR4 Chemokine Receptor Gene in Human CD34+ Hematopoietic Stem and Progenitor Cells for Engraftment in NSG Mice

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Ex vivo genetic manipulation and autologous transplantation of human CD34+ hematopoietic stem and progenitor cells (HSPCs) provides a promising therapeutic approach for a variety of blood and bone marrow disorders. However, for most disorders, genetic modification must confer a competitive advantage to the minority of corrected cells for clinical benefits to be observed. A recent report (Cell 160:686-699 [2015]) indicated that haploinsufficiency associated with monoallelic inactivation of the chemokine receptor cxcr4 gene may confer a competitive advantage to engrafting HSPCs. Hence, monoallelic disruption of cxcr4 by targeted insertion of a therapeutic gene within that locus may provide a growth advantage to the genetically modified cells. In this study, we used CRISPR/Cas9 technology to induce a haploinsufficient phenotype by indel formation at the cxcr4 locus of human CD34+ HSPCs isolated from healthy human donors, and evaluated the engraftment potential of these genetically modified cells in NSG mice. A 2'-O-methyl phosphorothioate-modified single guide RNA targeting exon 2 of the cxcr4 gene was chosen for the analysis. To generate Cas9-sgRNA ribonucleoprotein complexes (RNPs) for electroporation into recipient CD34+ HSPCs, the cxcr4-specific sgRNA was incubated with purified Cas9 protein in a 2.5:1 molar ratio prior to electroporation. G-CSF-mobilized CD34+ HSPCs isolated from healthy donors were electroporated with Cas9-sgRNA RNPs, or a Cas9-only control, and subsequently assayed for cxcr4 indel formation via T7 endonuclease I assay at three days post-electroporation. Using Cas9-sgRNA RNPs, we routinely observed an approximately 50% occurrence of indel formation with viability greater than 85% in human CD34+ HSPCs. To estimate the statistical distribution of monoallelic versus biallelic knockout of the cxcr4 gene under standard conditions, CD34+ HSPCs were electroporated with Cas9-sgRNA RNPs and plated in semi-solid medium in a colony forming unit assay. At approximately ten days post-plating, 30 well-isolated colonies were picked, and a portion of the cxcr4 locus was amplified by PCR. To score mono- and biallelic indel formation, PCR amplicons were subjected to Cas9-mediated cleavage in vitro. The frequency of knockout events fit a lognormal distribution ($R^2 = 1$), with monoallelic indel formation (52%) favored over either no detectable editing events (31%) or biallelic editing events (17%). To confirm partial knockout of cxcr4 at the protein level, we performed flow cytometric analyses of RNP-recipient cells. Cell surface expression of cxcr4 at three days post-electroporation demonstrated a pattern of mean fluorescence intensities consistent with the distribution of indel formation. To determine the effect of partial cxcr4 knockout on HSPC engraftment, NSG mice were injected with Cas9-sgRNA-recipient cells or cells that were electroporated with Cas9 only as a control. Murine bone marrow was harvested at 3 months post-transplantation and assayed for human cell engraftment via flow cytometry to detect CD45+ human cells. Preliminary results showed equivalent levels of engraftment of cxcr4-targeted CD45+ HSPCs and control CD45+ HSPCs (6.5% CD45+ cells and 5.5% CD45+ cells,

respectively) despite the estimated loss of total cxcr4 activity in ~17% of bulk cells in the cxcr4-targeted group. Studies aimed at isolating a population of CD34+ cells enriched for monoallelic disruption of cxcr4 are in progress to further investigate the competitive advantage of these cells.

637. CRISPR/Cas9-Mediated *In Vivo* Gene Targeting Corrects Hemostasis in Newborn and Adult Hemophilia B Mice

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CRISPR/Cas9, a powerful genome editing tool, is promising for efficient correction of disease-causing mutations. We recently developed a dual-AAV vector approach and demonstrated successful correction of a mutation in 10% of liver cells in a mouse model of ornithine transcarbamylase (OTC) deficiency following *in vivo* genome editing. However, most monogenic genetic diseases, including hemophilia, are caused by many different mutations scattered throughout a gene rather than a single predominant mutation. The vector developed for one mutation would not benefit a patient with a different mutation. In this study, we aimed to develop a universal CRISPR/Cas9 gene targeting approach in which the vector system could be applied to majority of patients with a specific disease, such as hemophilia B. To validate this new approach, we performed the experiment in a factor IX knockout (KO) mouse model. In this two-vector approach, vector 1 expresses the SaCas9 gene from a liver-specific TBG promoter, the same as it was in our previous approach. The difference lies in vector 2. In addition to the sgRNA sequence expressed from a U6 promoter that specifically targets a region in the 5' end of exon 2 of murine FIX and 1.7-kb donor fragments, it also contains a partial human FIX cDNA sequence spanning the remaining exon 2 to exon 8 followed by the bovine growth hormone polyA inserted in between the left and right arms of the donor template. In addition, the partial human FIX cDNA is codon optimized and carrying the hyperactive FIX Padua mutation. Following double strand breaks generated by Cas9 and HDR, the partial human FIX cDNA would be fused to the 5' end of murine FIX, and a hybrid of murine-human FIX transcript would be expressed from the native murine FIX promoter. The control vector 2 contains everything except for the 20-nt target sequence. Co-injection of the two vectors with varying doses in newborn and adult FIX-KO mice resulted in stable FIX activity at or above the normal levels for 8 months. Eight weeks after the vector treatment, a subgroup of the newborn and adult treated FIX-KO mice were subjected to a two third partial hepatectomy, and all of them survived the procedure without any complications or interventions. FIX levels persisted at similar levels after partial hepatectomy, indicating stable genomic targeting. FIX-expressing hepatocytes were detected in liver samples collected at the partial hepatectomy and at the end of the study. Chimeric murine-human FIX transcripts were detected by RT-PCR only in mice treated with the targeted vectors. This study provides convincing evidence for efficacy in a hemophilia B mouse model following *in vivo* genome editing by CRISPR/Cas9.

638. Toolkit to Facilitate CRISPR-Mediated Transgenesis in the Adult Rat Brain

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The development of CRISPR technology has revolutionized genome engineering and expanded biomedical research fields. This powerful tool can be utilized in various ways, notably in the genetic customization of model organisms and the interrogation of gene function. The use of CRISPR in vivo has been described in murine models, but studies have yet to demonstrate its action in the rat brain. Herein, we describe multiple AAV-based methodologies to employ CRISPR genome editing within the central nervous system (CNS) of an adult rat. Using tyrosine hydroxylase (TH) as the target gene for our proof-of-concept, we simultaneously injected two AAVs, one encoding Cas9 and the other encoding one or two TH-specific guide gRNAs and a GFP reporter, into the midbrain of an adult rat. After 2 and 6 weeks, we observed reduced expression of TH throughout the midbrain and striatum, respectively, confirming that virally delivered CRISPR can alter gene expression in wild-type rats. Our lab has developed a repertoire of transgenic "driver" rats expressing Cre in specific neuronal subtypes, as well as a rat carrying a Cre-dependent Cas9 transgene at the Rosa26 locus (lsl-Cas9). By crossing the lsl-Cas9 rat to a DAT-iCre rat, we obtained progeny whose Cas9 expression is restricted to dopaminergic neurons. The injection of a single AAV carrying a gRNA and GFP reporter into these rats resulted in effective knockdown of the target gene that was not observed in similarly injected wildtype rats. In conclusion, we present the first characterization of CRISPR-mediated genome modification in the rat brain as well as the production of a Cre-dependent Cas9 transgenic rat. These tools can be applied to investigate genes of interest in rat models of disease. The availability of rats expressing Cre-dependent Cas9 will reduce the variability and costs associated with these experiments, as only a single AAV is required to deliver gRNA to a region of interest.

639. Induction of Fetal Hemoglobin Synthesis in Human Erythroblasts by CRISPR/Cas9-Mediated Editing of the β-Globin Locus

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β-thalassemia and Sickle Cell Disease (SCD) are severe anemias caused by mutations in the β-globin gene cluster. Naturally occurring, large deletions in the β-globin locus result in increased fetal hemoglobin (HbF) expression (HPFH, Hereditary Persistence of Fetal Hemoglobin), a condition that mitigates the clinical severity of β-hemoglobinopathies. Here, we integrated BCL11A and GATA1 transcription factor binding site analysis and HPFH mutational data to identify potential HbF silencers in the β-globin locus. Based on this analysis, we designed a CRISPR/Cas9 strategy to disrupt: (i) a putative δγ-intergenic HbF silencer binding BCL11A and GATA1 in adult erythroblasts; (ii) the shorter deletion associated with elevated HbF levels ("Corfu" deletion) in β-thalassemic patients, encompassing the putative δγ-intergenic HbF silencer; (iii) a 13.6-kb genomic region including the δ- and β-globin genes and the putative intergenic HbF silencer. Targeted deletion or inversion of the 13.6-kb region caused a robust re-activation of HbF synthesis in an adult erythroid cell line, whereas deletion of the Corfu region and the putative δγ-intergenic HbF silencer failed to induce γ-globin expression. We then tested re-activation of HbF synthesis in primary hematopoietic stem/progenitor cells differentiated towards the erythroid lineage. Targeting the 13.6-kb genomic region resulted in a high proportion of γ-globin expressing erythroblasts, increased HbF synthesis, and amelioration of the sickle cell phenotype. These data suggest that the 13.6-kb region could serve as target for therapeutic genome editing for HbF induction in β-hemoglobinopathies. Overall, this study contributes to the knowledge of the mechanisms underlying fetal to adult Hb switching, and provides clues for a genome editing approach to the treatment of SCD and β-thalassemia.

640. CRISPR/Cas9 sgRNAs Do Not Exert the Same Competition with Liver microRNAs as shRNAs

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The ability to edit genomes using CRISPR/Cas9 machinery has piqued interest into whether this technology can safely be used for therapeutic purposes. We previously reported on the adverse consequences of increasing amounts of recombinant adeno-associated viruses (rAAVs) expressing small hairpin RNAs (shRNAs) driven by the U6 polymerase-III promoter and the effect on the miR-122 microRNA in the liver. This causes high levels of serum alanine aminotransferase (ALT), hepatocyte turnover and loss of rAAV genomes impairing the sustainability of shRNA expression ultimately leading to morbidity in the mice. This occurred irrespective of whether a host target was present. The prototypical CRISPR construct also requires high expression and typically has utilized the rAAVs as vectors for delivery and the U6 promoter to drive single guide RNA (sgRNA) expression. The composition of an ~100nt sgRNA differs from an shRNA and includes a target-specific 20nt CRISPR (crRNA) sequence followed by a ~80nt transactivating RNA (tracrRNA). To evaluate the effects of high levels of sgRNAs, we generated various rAAV-U6-sgRNA constructs with and without endogenous targets in the mouse. When tested in 293 cells these constructs generated abundant levels of sgRNAs but interestingly also led to the biogenesis of a ~25nt small RNA sequence enriched in the absence of co-administered Cas9. This additional small RNA species could have the potential to act in the microRNA machinery or otherwise disrupt cellular processes. We then tested *in vivo* consequences by delivering the rAAV-U6-sgRNA constructs to both wildtype C57Bl/6 mice and mice transgenic for Cas9 at the same dose and delivery method as AAV constructs bearing shRNAs. Importantly, toxicity - as measured by ALT levels over time and sustainable expression of sgRNAs - was not observed, nor was there competition with miR-122 or its various isoforms. We also did not detect the presence of ~25nt small RNA species in mouse liver samples. Steady state levels of the sgRNA was dependent on the presence of Cas9 and was able to edit over 50 percent of liver genomes one week after delivery of $5x10^{11}$ genome copies of rAAV-U6-sgRNAs in Cas9 transgenic mice. We are working towards identifying the degree of on-target and genome-wide off-target editing that occurs over various time points after sgRNA delivery. These results indicate that the general premise of CRISPR therapy design does not lead to similar detrimental consequences observed shRNAs, though various consequences of this approach still need to be evaluated and followed to ensure long-term safety and efficacy.

641. UDiTaS™: A Streamlined Genome Editing Detection Method for On- and Off-Target Edits, Large Deletions, and Translocations

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Genome editing technologies, including the CRISPR/Cas9 system, allow for precise and corrective DNA modifications to treat the underlying cause of genetic diseases. Development of accurate measurement technologies with nucleotide resolution is a key component of successfully translating these therapies to the clinic. We have developed a **U**ni-**Di**rectional **Ta**rgeted **S**equencing methodology, **UDiTaS**™, for simultaneous measurement of on-target editing, off-target editing, large deletions, translocations, and other structural changes. The multiplex compatible method improves on previous uni-directional amplification techniques (eg: AMP-Seq and HTGTS), implementing a robust and rapid tagmentation step with a custom designed Tn5 transposon. Superior quantification and throughput is achieved, in part, by incorporating unique molecular indexes for single molecule quantification and sample indexes for pooling.Editing events not possible to measure using standard PCR amplification with sequencing or digital droplet PCR (ddPCR) are now measurable across a >3 log range. Controlled spiking experiments with intra- and inter- chromosomal alterations allow for determination of reliable lower limits of detection and accurate measurement of editing events. UDiTaS has successfully detected a known translocation, a designed 1

kb deletion, small insertions and deletions, and, in addition, can find *de novo* translocations at endonuclease cut sites. Finally, multiplexing loci in a single reaction is being tested and, if successful, will enable parallel monitoring of many off-target editing sites when there is limited sample material, such as from *in vivo* studies.In summary, UDiTaS is a novel and robust sequencing method within the genome editing lab that has tremendous value for the translation of genome editing therapies. Insights gained from simultaneous measurement of small and large editing events, nucleotide resolution, and accurate quantification will augment selection and safety discussions.

642. Clinical-Meaningful Level CRISPR-Oligomer-Mediated Correction of Sickle Cell Disease (SCD) Using Non-Viral, cGMP Compliant, Scalable, and Closed System

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The A>T mutation in the first exon of the β-globin gene is the defining SCD mutation leading to all pathophysiological consequences. Currently, hematopoietic stem cell transplantation is the only cure for SCD. Unfortunately, most SCD patients don't have a matched donor. Novel approaches to treat SCD are still needed. Ex vivo viral vector based gene transfer and BCL11a knock-down to allow γ-globin expression are under evaluation. However, HDR-mediated gene correction is more appealing, permitting the corrected endogenous gene expression at its natural regulatory locus. Gene correction would also result in reduction of HbS concentration due to the introduced Indels, which could affect the propensity for sickling as demonstrated that the delay time to gelation is dependent of the 30th power of deoxyhemoglobin S concentration. Thus small changes in HbS concentration can theoretically have profound consequences. Correction of the SCD mutation is shown to be feasible. However, reported correction rates remain too low to reach the required target of ≥20% cellular correction, either double or single allele correction. With the successful development of efficient correction of monogenic "hotspot" mutation in CYBB gene in X-CGD, we report here the result of the efficient correction of another mutation, the SCD mutation, using a highly efficient CRISPR (Cas9 and sgRNA) system with donor oligomer, employing MaxCyte's commercially/clinically validated cGMP/regulatory compliant and closed platform technology. After screening candidate gRNAs, we optimized transfection conditions using an EBV-transformed B cell line (B-LCL) derived from an adult SCD patient as an initial model system. Transfection does not have long-term toxicity on B-LCL in viability and cell proliferation. For ease of analysis in process development, we integrated a 6 nucleotide HindIII-recognition site at the SCD mutation. We observed efficient site-specific integration of the HindIII-marker as evidenced by 1) HindIII digestion of the PCR-amplified genomic DNA (~50%) and 2) sequencing (~30%). Importantly, this optimized process yielded correction of ~30% of SCD patient B cells into wild type genotype with respect to the SCD mutation. To optimize and evaluate the correction rate using this technology in HSCs, we used a converse targeting similar to that we developed previously to overcome limited access to patient HSC. Specifically, we designed gRNA targeting healthy cells at the exact site corresponding to that of SCD mutation site, which represents a single nucleotide difference, so that the healthy cells could be converted into SCD cells. This approach is just the opposite process of correcting SCD mutation in patient HSC into health HSC. In theory, the converse rate from healthy HSC to SCD mutation HSC should be the same as correcting SCD patient HSC into healthy HSC. When applied to healthy cells, the biallelic conversion rate reached as high as ~30% by sequencing. Given that a single allele correction would result in the benign carrier state, quantitation of the clinically relevant cellular conversion rate could be 1.5x higher. This conversion rate, plus the additional ~30% Indels that could decrease HbS expression substantially, and the high rate of engraftment of corrected HSC observed from our previous study in gene correction in X-Linked CGD puts these results within the therapeutic window of reversing SCD. We are currently testing correction of SCD patient HSCs for both in vitro detection of WT globin expression in red blood cells differentiated from the corrected HSC and function in vivo in NSC mice engrafted with gene edited HSC.

643. Targeted Epigenetic Modifications as a Therapeutic Approach to Treat HIV Infection

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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. We observed a significant reduction in *CCR5* gene expression

as determined by quantitative RT-PCR seven days after the delivery of T-DEM-encoding mRNAs and a corresponding decrease in CCR5 cell surface levels assessed via flow cytometry. Next-generation bisulfite sequencing revealed up to 27% increase of CpG methylation at the target site compared to controls. Importantly, no changes in methylation profile could be detected at the top 10 predicted off-target sites and no significant changes in gene expression could be detected at the closest neighboring genes. 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.

644. Development of a Qualifiable MiSeq Assay for Precise and Accurate Quantitation of Small Insertions and Deletions (Indels) in the Human Genome Induced by Sequence-Specific Zinc-Finger Nucleases

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Genome editing offers a modality for gene therapy that is advancing rapidly toward clinical utility. Many applications use engineered nucleases to perform targeted gene disruption through induction of indels via non-homologous end joining. Monitoring efficiency of such editing events requires accurate and precise measurements of the proportion of cells that acquire indels at the intended site, which is monitored using Next Generation (Deep) Sequencing. Well-characterized quantitative assays will be required to adequately assess clinical results. The determination of parameters of precision, accuracy, and reproducibility used in pharmacokinetics (PK) assays of protein analytes is well-established in various publications, American Association of Pharmaceutical Scientists white papers, and FDA guidance documents. These principles have not previously been applied to indel quantitation. We have outlined a method for qualifying such assays using the Illumina® MiSeq platform and a proprietary in-house bioinformatics pipeline to identify and score indels. Indel-containing genomic DNA samples were produced by transduction of test human cell lines with mRNA encoding zinc finger nucleases (ZFNs) to induce mutations in a target gene. The indel levels evaluated by MiSeq were consistent with the percentage of clones shown to have indels using Sanger sequencing as an orthogonal method. To assess precision and accuracy across the entire dynamic range of quantitation, cell lines containing specific ZFN-induced mutations in every cell were cloned. Calibrator samples with known levels of indels were produced by mixing genomic DNA from ZFN-modified cell lines (with 100% indels) and from GFP mRNA-electroporated cells at various controlled percentages. Amplicons surrounding the sequence targeted for mutation were produced using 2 sequential PCR amplifications that in addition introduced sequence elements required for analysis by MiSeq. Genomic DNA and PCR products were precisely quantitated using well-characterized, qualified assays to assure reproducibility. High levels of genomic DNA were used in the initial PCR amplification to minimize binomial selection error. Reproducibility, precision, and accuracy were assessed using technical replicates at each stage of the workflow, and were affected by the levels of indels present as well as the amount of input genomic DNA. Acceptability of analytical recoveries was defined according to FDA guidance documents used in PK assays. Acceptable analytical recoveries (80-120%; or 75-125% for lowest calibrator) and %CV (<20%; <25% for lowest calibrator) were attained with log-log fit of the data. Linearity extended to ~0.1% indels. Analytical recoveries were much poorer using a linear-linear data model, with acceptability extending only down to ~2% indels. Variability between independently prepared technical replicates was <5% at each step of the workflow. Contrary to expectation, precision and accuracy in MiSeq quantitation at low levels of indels requires the use of a calibration curve. The results indicate that a qualifiable quantitative indel assay can be developed using MiSeq analysis.

645. High Specificity and Enhanced HDR Efficiency CRISPR/Cas9 Variants for Precision Genome Engineering

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CRISPR (Clustered regularly interspaced short palindromic repeats) Cas9 nucleases have revolutionized the field of genome editing enabling unprecedented efficiency of gene targeting in a vast array of cell types and organisms. The potential therapeutic applications of this technology are restrained by low HDR (Homology directed repair) efficiency and off-target effects. We are the pioneer on using single-stranded DNA oligonucleotides (ssODNs) for successful genome editing (HDR). Here we show our continuous efforts in optimizing ssODN design for CRISPR; our results provided definitive guidelines on maximizing HDR efficiency with CRISPR-ssODN system. Moreover, we independently designed Cas9-Geminin variants (the patent application was submitted in 2015); we demonstrated that Cas9-Geminin's expression was cell-cycle dependent and it significantly increased HDR/NHEJ ratio. eSpCas9 variant was reported to have high specificity. CRISPR RNP (ribonucleoproteins) has the potential to decrease the off-target effects as well. We generated the recombinant eSpCas9 proteins, and demonstrated that eSpCas9 proteins, in conjunction with advanced synthetic gRNA design, greatly reduced the off-target effects to undetectable level, while keeping the same on-target cleavage activities as wild type SpCas9 does. We provide programmable robust tools for efficient and precise genome engineering. Such advances have the potential to benefit human health by enabling new platforms for gene therapy and cell therapy.

646. Optimizing Homology-Directed Repair at the β-globin Locus to Correct Sickle Cell Anemia

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Sickle cell anemia is a monogenic disorder with an autosomal recessive inheritance. It is caused by a single nucleotide transversion that increases the hydrophobicity of adult globin (β^A) and renders it

susceptible to polymerization, resulting in the characteristic sickling pattern of erythrocytes. The symptoms manifests soon after birth when the globin expression switches from fetal to adult globin. The sickle globin (β^s) drives various sequelae such as anemia, hemolysis, vaso-occlusion, pain, organ damage and pre-mature death. We have developed and optimized gene editing strategies with various nucleases including TALENs as well as CRISPR/Cas9 methods using chemically modified single guide RNA and Cas9 protein to generate targeted double strand breaks at the sickle mutation in mobilized peripheral blood CD34+ cells. To facilitate homology-directed repair at the editing site, we have designed and screened rAAV6 repair templates which insert a known anti-sickling hemoglobin gene under the control of various regulatory elements. The efficiency of editing has been carefully characterized by digital droplet PCR (ddPCR) and TIDE sequencing to measure INDEL rates. Successful integration of our repair templates by homologous recombination has been validated using flow cytometry and ddPCR. The preservation of erythroid differentiation potential of edited human CD34+ cells was confirmed in culture and HPLC analysis of the differentiated erythrocytes demonstrated the presence of anti-sickling hemoglobin protein. Our study emphasizes the performance of various repair templates as well as monitoring the INDEL/HDR ratio as an important parameter in developing gene editing strategies to cure sickle cell disease.

647. Viral and Non-Viral Delivery of the CRISPR-Cas9 System in Human Hematopoietic Stem and Progenitor Cells

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Autologous transplantation of genetically corrected hematopoietic stem cells (HSC) is an attractive therapeutic alternative for patients with genetic blood diseases lacking a compatible HSC donor. This study aims at developing an efficient targeted therapy for β-hemoglobinopathies. The CRISPR-Cas9 system is a suitable tool for reproducing naturally occurring large deletions (Hereditary Persistence of Fetal Hemoglobin) in the β-globin locus, which result in beneficial expression of fetal hemoglobin (HbF). Here, we aimed at optimizing and comparing the efficiency of plasmid-, lentiviral (LV)-, RNA- and ribonucleoprotein complex (RNP)-based methods to deliver the CRISPR-Cas9 system to CD34+ hematopoietic stem/progenitor cells (HSPC). We selected specific gRNAs generating HPFH-like targeted deletions in the β-globin locus and provided proof of concept for this therapeutic approach by plasmid delivery of CRISPR-Cas9 in human HSPC, achieving around 35% of deletion/inversion in populations enriched for edited HSPC. HbF re-activation was observed at both mRNA and protein levels in HSPC-derived erythroid precursors. Since DNA transfection shows high toxicity in HSPC, we optimized and compared alternative CRISPR-Cas9 delivery systems. Transduction of HSPC using LVs expressing Cas9 and gRNA pairs was minimally toxic and efficient, but resulted in poor deletion and inversion efficiency (3% and 3.5%, respectively). We then tested DNA-free, non-viral RNA and RNP delivery methods to achieve efficient genome editing rate, while minimizing cytotoxicity and provide transient expression of the CRISPR-Cas9 system. We optimized several parameters, such as nucleofection program, cell concentration, RNA/RNP amount and cell culture conditions to maximize CRISPR-Cas9 delivery and HSPC viability. Both RNA and RNP delivery methods were highly reproducible, with some donor-related variability, and resulted in up to 12% deletion and 6% inversion events in bulk unselected HSPC. Overall, Cas9 RNP delivery exhibited a good balance between cytotoxicity and efficiency of genomic modifications as compared to the other delivery systems.

648. Designer Recombinases for Genome Surgery Recombinases for Genome Surgery

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Many genetic mutations that cause human diseases have been identified over the last decades. Recent breakthroughs in the field of genome editing now provide a genuine opportunity to establish innovative gene and cell therapy approaches to repair DNA lesions to replace, engineer or regenerate malfunctioning cells *in vitro*, or directly in the human body. However, most of the recently developed genome editing technologies introduce double stranded DNA breaks at a target locus as the first step to gene correction. These breaks are subsequently repaired by one of the cell intrinsic DNA repair mechanisms, typically inducing an abundance of random insertions and deletions (indels) at the target locus. Ideally, therapeutic genome editing should, however, be efficient and specific, without the introduction of indels. Site-specific recombinases (SSRs) allow genome editing without triggering cell intrinsic DNA repair pathways as these enzymes fulfill both cleavage and immediate resealing of the processed DNA, allowing precise, predictable and efficient genome editing in vivo. We use substrate-linked directed evolution coupled with rational design to program SSRs to target therapeutically relevant human genomic sites. Examples of our work will be presented, including the development and application of the broad-range anti-HIV-1 recombinase, Brec1. Because Brec1 efficiently and specifically eradicates the integrated HIV-1 provirus in humanized patient derived xenograft mouse models, it represents a promising new agent with curative potential.

649. Gene Editing ALS Causing $(GGGGCC)_n$ Repeat Expansion in C9orf72 Using CRISPER/Cas9 System

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ALS is a progressive neurodegenerative disease that affects motor neurons in patient's brain and spinal cord. Currently there are around 20,000 patients in the US with more than 5000 new cases diagnosed every year. Most people who develop ALS are between the ages of 40 to 70 years old, but several cases have been reported in much younger individuals. ALS prognosis is very poor with most patients dying within

5 years of diagnosis. ALS occurs in two forms: sporadic and familial. Sporadic ALS happens randomly within the population and accounts for 90-95% of all cases, while familial ALS is the inherited form of the disease. In 2011, a $(GGGGCC)_n$ repeat expansion in the non-coding region of the C9orf72 gene was identified as the major cause for both familial (25-40%) and sporadic (7%) ALS. Since then, three major hypotheses have emerged to explain how this expansion can cause ALS: 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. 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 with ~20 nts of complementarity. Current gene therapy approaches being developed with CRISPR/Cas9 involve delivery of the Cas9 enzyme with a guide RNA 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. We have designed several strategies to target the repeat expansion in the intronic region without affecting any of the exons. We have successfully designed and tested guide RNAs in Hek-293 cells that were able to remove the repeat region using CRISPR Cas9 system. We then packaged these RNA guides into rAAV9 vectors for invivo delivery. Here we will show some data to suggest that gene editing is occurring in primary neurons in culture and in animals in vivo through tail vein injections.

650. Characterizing the Mutational Limits of *S. pyogenes* Cas9

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Site-specific nucleases have been harnessed as powerful tools for gene editing in a diverse set of organisms. The most flexible of these, RNA-guided endonucleases (RGENs), provide the most accessible nuclease platform due to their simplicity. As the number of RGENs available increases, rapid methods to refine their function will accelerate the transition into potent therapeutics. Toward this end, we have defined the spectrum of mutation tolerance of *S. pyogenes* Cas9 using deep mutational scanning (DMS). We employed a nuclease screen in bacterial cells to apply selection pressure to a library of Cas9 mutants encompassing over 8500 nonsynonymous mutations. Following selection, deep sequencing revealed changes in mutation frequency which correlate with nuclease activity. As expected, less conserved domains tolerate more mutations than the catalytic nuclease domains. In addition, this approach identified specific amino acids critical to the function of the enzyme and amino acid mutations which enhance the nuclease activity. The mutation profile generated in this work offers a solid foundation for future Cas9 protein engineering. Furthermore, the mutational constraints uncovered here present insights into unknown, potentially important mechanistic features of the nuclease which warrant further study. Our work demonstrates how applying DMS to novel, emerging RGEN technologies can increase the rate of nuclease refinement which should facilitate their adoption into gene therapy applications.

651. Correction of Multiple Cystic Fibrosis-Causing Variants by CFTR Superexon Homology-Independent Targeted Integration

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Cystic Fibrosis (CF) is a recessive disorder caused by mutations in both alleles of the CFTR gene. Approximately 90% of individuals with CF carry at least one allele with the c.1521_1523delCTT variant, commonly known as F508del, but there are more than 270 other disease-causing variants. The use of ZFN, TALEN and CRISPR 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). A recent study to establish proof-of-principle for correction of multiple mutations with a single superexon donor via HDR was successful (Bednarski, 2016) but the efficiency was very low. Here we describe the incorporation of two different superexons into the CFTR locus using Cas9/gRNA homology-independent targeted integration (HITI), a recently described technique (Suzuki et al., 2016) that significantly increases gene editing efficiency. The HITI method takes advantage of the fact that if the superexon is integrated in the wrong orientation, then in the majority of cases, the gRNA will excise it and recreate the target site for a subsequent attempt at correct integration without the need for additional manipulation. In addition to higher editing efficiency, HITI allows editing of 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 comprise an inverted gRNA target site, a splice acceptor site and either superexon sequences of exons 11-27 or 23-27 and a 2A-mCherry reporter gene. The constructs have been designed to be incorporated into intron 10 or 22 respectively, using Cas9/gRNAs previously validated in our lab (Sanz, 2016). Successful integration of the CF Superexons 11-27 or 23-27 should potentially correct 92% or 5% of CFTR variants respectively. The chimeric pre-mRNA is predicted to conserve many of the normal regulatory features of the intact gene including the promoter and the intronic regions upstream to the integration site. Bednarski C (2016). PLoS One **11**:e0161072. Hollywood J (2016). Sci Rep. **6**:32230. McNeer N (2015). Nat Commun. **6**:6952. Sanz D (2016). **13th** ECFS Basic Science Conference, Pisa Suzuki K (2016). Nature **540**:144-149.

652. A Marker-Free Co-Selection Strategy for High Efficiency Human Genome Engineering

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Targeted genome editing using engineered nucleases facilitates the creation of *bona fide* cellular models for biological research and may be applied to human cell-based therapies. Broadly applicable and versatile methods for increasing the levels of gene editing in cell populations remain highly desirable due to the variable efficiency between distinct genomic loci and cell types. Harnessing the multiplexing capabilities of the clustered, regularly interspaced, short palindromic repeat (CRISPR)/Cas9 system, we designed a simple and robust co-selection strategy for enriching cells harboring either nuclease-driven non-homologous end joining (NHEJ) or homology-directed repair (HDR) events. Selection for dominant alleles of the endogenous sodium-potassium pump (Na^+,K^+-ATPase) that render cells resistant to ouabain is used to enrich for custom modifications at another unlinked locus of interest, effectively increasing the recovery of engineered cells. The level of improvement for targeted integration of transgenes reached 26 fold and the process was readily adaptable to transformed and primary cells, including hematopoietic stem and progenitor cells (HSPCs). The use of universal CRISPR reagents and a commercially available small molecule inhibitor streamlines the incorporation of marker-free genetic changes in human cells.

653. Epigenetic Enhancement of Plasmid Gene Therapy

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Since plasmids are easily manipulated and purified, they have become the vector of choice for gene delivery. However, recent studies have shown that plasmids are susceptible to epigenetic silencing over time. Specifically, transgene expression can be regulated by modification of N-terminal amino acid tails of histone proteins, which tightly bind DNA and alter the accessibility of plasmids for transcription. Several of the histone modifications associated with transcriptional inactivation (e.g. methylation of lysine 9 on histone 3; H3K9me) and activation (e.g. acetylation of lysines; H3K9ac) have been observed with plasmids. For example, results reported by Maniar et al. *Mol. Ther.* 21, 131-8 (2013) have shown different levels of modifications associated with plasmid DNA for activation (e.g. a decrease in H3K4me3 and H3K36me3) and silencing (e.g. an increase in H3K9me3 and H3K27me3). The goal of this work is to insert binding sites for known histone modifying enzymes into the plasmid DNA sequence near promoters to enhance transgene expression by manipulating epigenetic regulation. To accomplish this goal, transcription factor binding sequences (TFBS) were cloned upstream and downstream of the promoter region and tested for their effect on transgene expression in prostate cancer cells (PC3). In addition, the effect of TFBS copy number was also studied. Enhancer effects were tested on two different plasmids (maps shown in Figure 1) containing the luciferase reporter gene: one with the human EF1α promoter (pEF-LUC) and one with the viral CMV promoter (pCMV-LUC). The sequences used in this study (see Table 1) were specifically chosen since they are known to impart beneficial histone modifications, including H3K9ac, H3K4me3, H3K4me1, and H4R3me. Lead transcription factor binding sites providing < 2-fold increases in relative luminescence relative to the control with the EF1α promoter have been identified: NFY/NF-1, GFY, and HNF4. Interestingly, a shared consensus sequence emerged within the TFBS leads: $CaAttN_{1-2}CCA$. Leads were not identified with the CMV promoter since some TFBS are already present within the promoter yet absent in the EF1α promoter. Chromatin remodeling effects were quantified via chromatin histone immunoprecipitation (ChIP) to measure levels of specific histone markers such as H3K9ac (activation) compared to control plasmids lacking enhancers. Ongoing studies include quantifying increases in nuclear uptake via nuclear isolation/qPCR and visualization of plasmid uptake and location within transfected cells with fluorescence in situ hybridization (FISH) to determine if the TFBS also enhances nuclear uptake. Overall, these results demonstrate that the addition of a single TFBS to a plasmid can significantly increase transgene expression via epigenetic modifications.

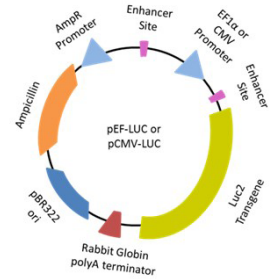

Figure 1 – Plasmid Structure

Table 1 – Enhancer/Transcription Factor Binding Sequences

Histone Modification	Enhancer Enzyme/TF	Abbrev.	DNA Binding Sequence
Acetylation	ATF2	AT	TGACGTCA
	(p300/CBP)-SP1	SP	GGGGGCGGGGC
	(p300/CBP)-HNF4	HN	CAAAGTCCA
	(p300/CBP)-Zta	ZT	GATCTTCTAGACCAAATGTGCAAAGGTGAG
	POU6F1	PO	AATGAG
	VSX2	VS	AATTAG
H3K4me1	AP-1	A1	ATGACTCATC
	AP-2a	A2	ATGCCCTGAGGC
	MYB (HTH)	MY	GCGGTTA
H3K4me3 H3K9ac	NFY/NF-1 (1)	NF	ATTGGCTATTGGCCAT
	NFY/NF-1 (2)	NF2	ATTGGCCAATCGCCAT
	GFY-Staf	GF	AACTACAATTCCCAGAATGC
H4R3me	(Yin-Yang TF)-DRBP76	YY	CGCCATATT
Chromatin Remodeling	Androgen Receptor	AN	GGAACACGTGTCTTCT
	CTCF	CT	CCGCGAGGAGGCAG
	Poly(dA:dT)	PA	AAAAAAAAAAAAAAAA

654. Targeted Genome Editing of Recombination Activating Gene 1 to Potentially Treat Severe Combined Immunodeficiency

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Recombination-activating gene 1 (RAG1) encodes a lymphoid-specific protein that is expressed during the early stages of T cell and B cell development and, together with *RAG2*, initiates the process of V(D)J recombination by which the wide repertoire of antigen-specific T and B receptors is generated. Mutations in RAG genes are associated with a broad spectrum of clinical phenotypes in humans, ranging from severe combined immunodeficiency (SCID) associated with a lack of circulating T and B cells, to autoimmune phenotypes such as in Omenn syndrome. The treatment of these patients remains critical given that HSC transplantation from related, partially HLA-matched donor, is often associated with unsatisfactory clinical outcome. On the other hand, critical safety concerns related to ectopic and unregulated Rag1 expression has so far hampered conventional gene replacement with randomly integrating vectors. To overcome this hurdle, we propose to take advantage of a specific genome editing strategy, based on the delivery of engineered nucleases and DNA template, to directly correct *RAG1* mutations and restore both gene function and expression control without the risk of insertional mutagenesis. We are testing different protocols to deliver in human cell lines and CD34+ cells a *RAG1* gene donor vector together with Zinc Finger Nucleases (ZFN). Our preliminary results show good levels of ZFN activity (measured as frequency of NHEJ-mutagenesis) and targeting efficiency (measured as GFP expression) when ZFNs are delivered 1 day after the donor transduction both in cell lines and CD34+ cells. In parallel we are setting up a specific molecular assay as a tool to evaluate targeted integration in human *RAG1* locus. These preliminary results are instrumental to further optimize the genome editing protocol for *RAG1* correction, which will be used on CD34+ cells derived from patients.

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655. Targeted FVIII Expression Under the Control of Its Native Promoter for Hemophilia A Gene and Cell Therapy

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We studied the activity of F8 promoter (pF8) sequence to drive transgene expression in a LV construct to verify the feasibility of expressing FVIII under its natural promoter for gene therapy approaches. 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. We validated some of these TF by a luciferase specific assay and found some endothelial-specific TF able to activate F8 promoter activity. After LV.pF8.GFP injection in C57Bl/6 mice, GFP expression was restricted to liver sinusoidal endothelial (LSEC). Overall, this work provides new insight in extrahepatic sources of FVIII and in its transcriptional regulation. pF8 study showed its ability to drive transgene expression in LSEC, in splenic macrophages and, generally, in myeloid cells and less in lymphocytes by *in vivo* and *ex-vivo* LV delivery. Finally, by expressing FVIII under its promoter we were able to reach therapeutic levels of FVIII in HA mice without immune response.Instead, in spleen and bone marrow, GFP was in myeloid cells. We injected LV.pF8.hFVIII in C57/BL6 and 129/Bl/6 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 consistent blood loss reduction in gene-corrected hemophilic mice. To improve FVIII levels we generated LVs in which BDD-FVIII was substitute with more active forms of FVIII such as FVIII.RH and FVIII.N6 producing up to two fold more FVIII activity in hemophilic mice. Further, we transplanted LV-pF8.FVIII transduced human cord-blood CD34+ cells in busulfan-treated NOD/SCID gamma-null HA-mice. Human cells engrafted in mice showing a chimerism around 30% up to 4 months later. aPTT assay on plasma of LV-pF8-FVIII-CD34+ transplanted mice showed therapeutic levels of FVIII activity up to 10% of normal meanwhile transplantation of untransduced CD34+ cells reached 2% of activity as previously reported. Our results demonstrate that pF8 is differentially active in cell-subpopulations of several organs contributing to identify the FVIII producing cells and targeting FVIII expression in these cells by LV produced FVIII in therapeutic range in hemophilic mice. Overall, this work provides new insight in extrahepatic sources of FVIII and in its transcriptional regulation. pF8 study showed its ability to drive transgene expression in LSEC, in splenic macrophages and, generally, in myeloid cells by *in vivo* and *ex-vivo* LV delivery. Finally, by expressing FVIII under its promoter we were able to reach therapeutic levels of FVIII in HA mice without immune response.

656. Molecular Evidence of *Ex Vivo* Gene Editing in a Mouse Model of Immunodeficiency

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Gene editing is the introduction of directed modifications in the genome, a process boosted to therapeutic levels by the use of designer nucleases. Building on the experience of *ex vivo* gene therapy for severe combined immunodeficiencies, it is likely that gene editing of haematopoietic stem/progenitor cells (HSPC) for correction of inherited blood diseases will be an early clinical application. Here we show molecular evidence of gene correction in a mouse model of primary immunodeficiency. *In vitro* experiments in *Prkdc scid* fibroblasts using designed zinc finger nucleases (ZFN) and a repair template demonstrated molecular and functional correction of the defect. Following transplantation of *ex vivo* gene-edited *Prkdc scid* HSPC, definite proof of the ability of these cells to mediate some level of reconstitution has been obtained by deep sequencing of tissues from some of the recipients, which carried the expected genomic signature of ZFN-driven gene correction (correction of the *scid* mutation associated to the introduction of a *Bsa*WI diagnostic site). This signature has been observed in peripheral blood mononuclear cells (PBMC), thymus, spleen and purified spleen CD3 and CD8 T-cells. Levels are variable in the positive primary transplanted animals, higher with integration-proficient lentiviral vector (IPLV)-ZFN than integration-deficient lentiviral vector (IDLV)-ZFN, and were also observed in secondary recipients. In some primary and secondary transplant recipients we also detected double-positive CD4/CD8 T-cells in thymus and single-positive T-cells in blood, but no other evidence of immune reconstitution. We consider the presence of the genome editing signature proof that reconstitution can be achieved, albeit inefficiently both in terms of the number of positive animals and the levels in different tissues. This *Prkdc* model is deficient in non-homologous end-joining, and this deficiency may be responsible for the frequency of a recurrent 44-bp deletion including the ZFN target site that we have observed in multiple samples *in vitro* and *ex vivo*. This highlights the importance of considering the possible effect of DNA damage repair defects on gene editing strategies.

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H.H.A.-R. and C.R. contributed equally to this work

657. Evolution of HIV-1 Resistance to the Fusion Inhibitor C34-CXCR4 and Potential Fitness Costs in Consideration of a Phase 1 Clinical Trial

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We recently reported that when a 34 a.a. peptide (C34) from the HIV C-terminal heptad repeat-2 domain (HR2) of gp41 is fused to the N-terminus of CXCR4 (C34-X4) and stably transduced into primary CD4 T cells that broad and potent inhibition of HIV-1 infection of primary CD4 cells is observed both *in vitro* and *in vivo* in the NSG mouse model (Leslie G, et al PLoS Pathogens, 2016). C34-X4 expression confers protection from X4 and R5 isolates with high potency and specificity, and could also inhibit viruses resistant to the soluble HR-2 peptide inhibitor, enfirvutide. The inhibitory effects of C34-X4 when expressed in autologous primary CD4 T cells is currently being assessed in a Phase 1 clinical trial. To assess how HIV-1 might escape C34-X4 inhibition in vivo, we passaged HIV-1 isolates YU2 and JRFL (R5 tropic) and R3A (dual tropic) in mixtures of SupT1 cell lines that expressed CCR5 in the presence or absence of C34-X4. Viruses capable of replicating in C34-X4 expressing cells appeared after 43 passages for YU2, 35 passages for JRFL, and 21 passages for R3A. For all viruses selected, mutations in HR1 similar to those capable of conferring resistance to enfirvutide were observed, although additional changes in HR2 were also present. Interestingly, C34-X4 resistant YU2, JRFL and R3A all developed mutations in the viral Vpu protein (e.g. point mutations in the initiation methione or premature termination codons) that likely ablated its expression. Using infectious molecular clones containing Envs from C34-X4 resistant viruses with or without these Vpu mutations, we observed that viral growth was markedly enhanced by the absence of Vpu, and that when viruses initially contained Vpu, C34-X4 expression created strong selection pressures to ablate this gene. In addition to its ability to downregulate CD4, Vpu potently reduces expression of tetherin/BST2 in virally expressing cells. When unopposed by Vpu, tetherin has been shown to impede virion release from the cell surface resulting in the generation of large extracellular aggregates that have been proposed to facilitate cell-cell transmission of HIV-1 in some in vitro models. The extent to which loss of Vpu in the presence of tetherin and the formation of viral aggregates could contribute to C34-CXCR4 resistance is currently being evaluated. Because HIV-1 resistance to cell-associated C34-CXCR4 may require both changes in gp41 and loss of a critical viral restriction, these preliminary results suggest that there are likely to be strong selection pressures against the emergence of C34-X4 resistant viruses in vivo - a hypothesis that will be addressed in our upcoming clinical trial.

658. Preclinical Development of Gene Therapy for X-Linked Severe Combined Immunodeficiency (SCID-X1)

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X-linked severe combined immunodeficiency (SCID-X1) is caused by mutations in the gene encoding the interleukin-2 receptor γ chain (*IL2RG*), and is characterized by profound defects in T-, B- and NK-cell functions. Previous gene therapy clinical trials based on hematopoietic stem/progenitor cells (HSPCs) genetically corrected with MLV-derived retroviral vectors showed restoration of T-cell immunity but resulted in vector-induced leukemia through insertional mutagenesis. The use of an enhancer-less MLV vector to deliver the *IL2RG* gene caused no adverse events while retaining a significant clinical benefit. To increase the efficacy of gene therapy and further reduce potential genotoxicity, we developed a SIN lentiviral vector carrying a codon-optimized human *IL2RG* cDNA under the control of the human EF1α-S promoter. Codon optimization resulted in a 3-fold increase in mRNA and a 1.5-fold increase in protein expression per integrated vector copy. The performance of the vector was demonstrated *in vitro* by the restoration of a normal level of IL2RG mRNA or protein in a IL2RG-deficient T-cell line, patient-derived EBV-immortalized B-cells and mobilized $CD34^+$ HSPCs, with no impact on viability or clonogenic capacity. An *in vitro* immortalization assay (IVIM) showed a safe genotoxic profile, while the *in vivo* safety and efficacy of the vector was tested it in a preclinical model of SCID-X1 gene therapy based on transplantation of genetically corrected Lin^- cells from *IL2rg*$^{-/-}$ donor mice into lethally-irradiated *IL2rg*$^{-/-}$*-Rag2*$^{-/-}$ recipients. The study showed restoration of T, B and NK cell counts in bone marrow and peripheral blood, normalization of lymphoid organs (thymus and spleen) and a frequency of hematopoietic abnormalities comparable to that of control animals six months after transplantation. An extensive insertion site analysis carried out in bone marrow, thymus and peripheral blood of individual or groups of animals showed the expected genomic integration profile and no signs of clonal dominance in transduced cells. Interestingly, analysis of >100,000 integration sites in pre- and post-transplant murine cells showed that lentiviral vectors target at high frequency a substantially different set of genes compared to human $CD34^+$ cells, uncovering the limits in the predictive power of mouse-based genotoxicity studies. These studies will enable a multicenter phase-I/II clinical trial aimed at establishing the safety and clinical efficacy of lentiviral vector-mediated gene therapy for SCID-X1 in infants after non-myeloablative conditioning, and sustained restoration of both T- and B-cell immunity.

659. 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 Lentiviral Vector (LV) hematopoietic stem cell (HSC) gene therapy (GT), patient-derived HSCs are genetically marked by semi-random vector insertions which allow their stable genetic modification. Insertions are unique genetic marks that can be used as surrogate of clonal identity enabling the identification, tracking and monitoring of infected cell clones. Thus, integration studies provide important safety and efficacy readouts for any HSC-GT application. However, interpreting integration data can be difficult, as clones harboring integrations close to cancer related genes didn't always result in adverse events, nor clonal expansions were always a sign of ongoing tumor development.

To understand and improve the predictive potential of integration studies, we set-up an experimental framework that evaluates and compares the hematopoiesis after HSC-GT in sensitive tumor prone *Cdkn2a-/-* and wild type (WT) mice transplanted with HSCs transduced with a genotoxic (LV.SF.LTR) or a GT-like (SINLV.PGK) vectors. In this context, WT mice transplanted with *Cdkn2a-/- lin-* cells transduced with LV.SF.LTR (N=24) developed tumors earlier than mock-treated controls (N=19, $p<0.0001$), while WT mice receiving SINLV.PGK-transduced *Cdkn2a-/- lin-* cells (N=23) did not accelerate tumor onset. On the contrary, WT mice transplanted with WT *lin-* cells treated with LV.SF.LTR (N=25) or SINLV.PGK (N=24) did not develop tumors and have comparable survival curves. The dynamic of hematopoietic reconstitution was monthly evaluated by cytofluorimetric analyses of the blood of the transplanted animals. No major differences in the early phases of hematopoietic reconstitution were observed among the different group of transplanted animals, only at later time points major changes in blood composition could be observed in mice transplanted with *Cdkn2a-/- lin-* cells, where expanded clones started to be detected and followed in their growth over time.

Linker-mediated PCR on sonicated DNA was used to identify and accurately quantify the integration sites (IS) in monocytes, B and T cells isolated over time from the blood of the transplanted animals. Overall, we collected more than 80K ISs. Retrieval of IS from tumor is on-going. As preliminary result, we observed that independently from the genetic background of the transduced cells, mice transplanted with SINLV.PGK-treated cells were characterized by a highly polyclonal and diverse hematopoietic reconstitution over time, while mice receiving LV.SF.LTR-transduced cells showed a progressive reduction towards oligoclonality and a decrease in clonal diversity. Thus, our results revealed that a genotoxic and a GT-like vector have a different impact on the dynamics of hematopoietic reconstitution.

Our integration-based study will help unravelling the Darwinian rules driving clonal competition that occurs in the course of normal hematopoietic reconstitution and prior to malignant cell transformation.

660. Transduction of CMV-Specific T Cells with Anti-HIV CAR Vectors and Adoptive T Cell Immunotherapy in NHP Model

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Background: T cell (Tc) immunotherapy has been clinically successful in advanced ALL using genetically modified Tc to express CAR (chimeric antigen receptor) with a CD19 extracellular antigen-binding domain and intracellular Tc-signaling domain, which redirect CAR Tc to the tumor antigen. Tc immunotherapy for HIV infection would require homing to residual HIV reservoirs, effective killing of infected cells, and long-term persistence of CAR transduced Tc *in vivo*. These studies aim to stimulate CMV-specific Tc and genetically modify them with anti-HIV CAR vectors, and link HIV killing *in vivo* to the activation and persistence of CMV antigen presentation. Methods: Three rhesus macaque were challenged with SHIV-D, a CCR5-tropic SIV-HIV chimeric virus, prior to viral suppression with cART (TFV, FTC, DTG). Autologous rhesus PBMC were stimulated with αCD3/αCD28 coated beads or rhesus CMV-derived peptides pools and combinations of cytokines. The expansion of CMV specific Tc was determined by ELIspot and ICS (intracellular cytokine staining), and the phenotype analyzed by flow cytometry. CMV-specific Tc were then stimulated with CMV peptides and transduced with MLV-based CAR vectors (αCEA control or CD4-28ζ/maC46 fusion inhibitor). CAR Tc were expanded up to 120 x 10^6 cells and reinfused after structured treatment interruption (STI). Viral load and pharmacokinetics were measured by qPCR. Results: Optimized conditions stimulated up to 100-fold CMV-specific Tc expansion in two weeks; ICS confirmed expansion of CMV responsive Tc. Furthermore, $CD8^+$ Tc were efficiently transduced (~50%) with MLV-based vectors. After CAR Tc infusion and STI, viral load for the control was sustained at above $1x10^4$ copies/ml 3 weeks post, while viral rebound peaked and lowered to 1.2 x 10^3 in one animal and was delayed until week 6 in the other treated animal. PK values for the CAR Tc peaked one hour post infusion, with >80% decline in circulating CAR Tc at 24 hours. The circulating CAR Tc passed to undetectable levels (<300 copies/ml) in two animals (CEA control and one HIV CAR animal), and persisted at measurable levels in the circulation beyond 4 weeks in the second HIV CAR animal. Conclusions: Rhesus CMV-specific Tc were expanded and transduced with CAR vectors prior to adoptive Tc immunotherapy in the rhesus SHIV challenge model with cART. Transfer of genetically modified CMV-specific Tc were detected after 6 weeks and showed evidence of viral control. Expansion and long-term persistence of HIV-specific Tc may provide continuous immunosurveillance over HIV replication and residual HIV reservoir.

661. Genome Editing Based Reactivation of Fetal Hemoglobin Expression in HSPCs for Treating Sickle Cell Disease

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Sickle cell disease (SCD) is a genetic disease that affects millions of people worldwide. Although SCD can be cured by allogeneic hematopoietic stem cell transplantation (HSCT), this treatment strategy is only available to a small number of patients. It has been shown that elevated levels of fetal hemoglobin (HbF), such as those in persons with hereditary persistence of fetal hemoglobin (HPFH), may lead to improvement of the clinical presentation of patients with SCD, but hydroxyurea treatment, the current standard practice to induce HbF, has substantial limitations. Here we describe the use of CRISPR/Cas9 to induce large gene deletions that mimic HPFH and demonstrate that this approach results in reactivation of HbF expression in adult cells. Specifically, guide RNAs (gRNAs) targeting HPFH deletion junctions were pre-screened in 293T cells to identify highly active ones. The selected gRNAs were delivered into K-562 cells as pairs to induce large deletions of varying length. The frequency of deletion varied from <1% up to 42%. We found that the deletion frequency has no correlation with the size of deletion and a weak correlation with the activities of individual gRNAs. Optimal gRNA pairs were tested in both normal $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) and those from sickle cell disease patients. In HSPCs, HPFH deletions were observed in up to 20% of alleles. Treated $CD34^+$ cells were differentiated into erythroid cells and analyzed by HPLC and flow cytometry. Cells treated with HPFH gRNAs had a 2-fold increase in HbF protein levels as well as a 2-fold increase in the number of HbF positive cells. Unbiased off-target analysis of the gRNAs was performed using the GUIDE-Seq method. Of the five gRNAs analyzed, three gRNAs each had one active off-target site, but only one off-target site had activity above 1%. In summary, we demonstrate that well-designed CRISPR-Cas9 systems can induce HPFH deletions in HSPCs effectively, resulting in elevated levels of HbF with minimal off-target effects. This may serve as a novel therapeutic strategy for treating sickle cell disease.

662. AAV8-Mediated Targeting of Hepatocytes for the Treatment of Iron-Overload Induced Hepatic Inflammation

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Frequent blood transfusions among patients with thalassemia and sickle cell disease can commonly lead to iron-overload (IO), also known as transfusional hemosiderosis. Excess iron is largely accumulated in the liver, the primary organ for iron recycling and storage, and eventually leads to significant hepatic injury. These IO-induced damages are initiated by hepatic

inflammation, such as increased transaminases. It was generally thought that IO targets hepatic macrophages and produces reactive oxygen species, resulting in substantial inflammatory responses. MicroRNA-122, the predominant miRNA in hepatocytes, comprises up to 70% of the total hepatic miRNA population. It was concluded that microRNA-122 is a key regulator of multiple hepatic pathways in liver development, fat metabolism, tumorgenesis, and inflammation. Recently, decrease of microRNA-122 was observed in a genetic knockout (Hfe-/-) mouse model of IO (*J Clin Invest.* 2011; 121(4):1386-1396). Thus, it was of interest to examine whether hepatocytes, instead of macrophages, directly contribute to IO-mediated hepatic inflammation. Here, we report that in the mouse liver, IO resulted in altered expressions of not only inflammatory genes, but also more than 230 genes that are known to be targets of the microRNA-122. In addition, both in human hepatic cell lines in vitro and in mouse hepatocytes in vivo, IO led to up-regulation of hepatic inflammation, such as the CCL2/NFκB pathway. This up-regulation was associated with significant reduction of HNF4α and its downstream target microRNA-122. Similar results were obtained in both the mice with acute IO (injected with iron-dextran) and the mice with chronic IO (fed with iron-rich diet). Interestingly, the same signaling pathway was up-regulated in macrophage-deficient mice, suggesting that macrophages were not the only target of IO. More importantly, the enhancement of hepatic inflammation could be rescued, at least partially, by overexpression of microRNA-122 by an AAV8 vector in a hepatocyte-specific manner (Table 1). Our findings indicate the direct involvement of hepatocytes in the IO-induced hepatic inflammation and are informative for developing new molecular targets and AAV8-mediated therapies for patients with major hemoglobinopathies.

Table 1. Body iron indices and hepatic inflammation

	IA	IO	IO +rAAV	IO +rAAV -122
Serum iron (μM)	20.8 ± 5.7	42.2 ± 7.7	42.0 ± 6.7	36.9 ± 4.9
TIBC (μmol/L)	49.7 ± 2.5	53.5 ± 4.4	54.4 ± 3.8	52.5 ± 4.9
TS (%)	41.5 ± 9.6	78.3 ± 9.6	76.8 ± 8.0	70.4 ± 9.0
Liver iron (μg/g)	101.3 ± 47.6	1760 ± 273	1792 ± 435	1665 ± 138
ALT (U/T)	30.3 ± 6.5	49.8 ± 4.0	49.5 ± 2.5	35.5 ± 4.7
AST (U/T)	86.8 ± 4.3	131.3 ± 13.1	145.3 ± 28.9	89.5 ± 7.6
Hepatic Inflammation Score	0.25 ± 0.5	2.25 ± 0.5	1.75 ± 0.5	1 ± 0.8

663. Using CRISPR-Cas9 to Purge the Latent HIV Reservoir

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Since its identification in 1983, HIV has remained a global pandemic. Worldwide, an estimated 35 million people are currently living with HIV, and an estimated 39 million people have died of AIDS-related illness since the onset of the epidemic. Strict adherence to combination antiretroviral therapy (cART) has significantly improved the quality of life and life expectancy of many infected individuals, transforming HIV into a chronic, manageable illness in the industrialized world. However, while cART inhibits viral replication, it does not target the transcriptionally silent, but replication-competent, residual population of virus hidden within certain cells. This so-called latent reservoir is established when infected CD4 T cells revert to a resting memory state. The resulting cells are largely non-permissive for viral gene expression and therefore do not generate new viral progeny. Because latent HIV is refractory to both immunological surveillance and cART intervention, it poses a major obstacle to full viral eradication and a true cure. We have demonstrated that engineered transcriptional activation systems based on CRISPR-Cas9 can be harnessed to reverse HIV latency by stimulating gene expression from the HIV long-terminal repeat promoter. Additionally, we have used a genome-wide CRISPR-based gain-of-function screen to identify human genes that induce latent viral reactivation following their own activation via a Cas9-based transcriptional effector. These genes were not previously implicated in HIV pathogenesis but could effectively induce HIV expression in several *in vitro* models of HIV latency following lentivirus-mediated gene transfer. These results demonstrate that CRISPR systems hold potential for treating latent HIV infection.

664. Exploring the Influence of HIV-1 Integration in Different Tissues: Blood and Gut

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Background: Combination antiretroviral therapy (cART) can effectively control viral replication and normalize immune function. However, latent persistence of HIV-infected cells prevents full eradication of the virus. Therefore, HIV integration into the cellular genome can play a critical role in expansion and persistence of HIV-infected cells, as has been reported in recent research studies. Here, we studied proviral reservoir in blood and rectal tissue between different patient cohorts in order to reach new insights in the mechanisms of reservoir maintenance and propagation. Methods: Viral integration sites were identified in 15 patients of a cross-sectional study, enrolled in two clinical centers (Ghent, BE and London, UK) including three patient cohorts: early treated patients with ART started during seroconversion (Early ART, n=5), patients with late ART initiation during chronic phase of HIV-1 infection (Late ART, n=5) and long-term non-progressors (LTNP, n=5). Patients within Early ART and Late ART cohorts were treated uninterrupted for a median of 10 years with undetectable viral load for at least 4 years. Viral integrations were determined by LAM-PCR both in peripheral blood mononuclear cells (PBMCs) and gut tissue (rectal biopsies). Results: A total of 2326 IS were obtained from PBMCs or rectal biopsies from the 15 patients, being composed of 2171 different integration events. Additionally, we also found that more than 7% (155) shared IS were observed between the PBMCs and rectal biopsies. Integrations into the BACH2 and MKL2 gene regions, respectively, were found in two samples. These gene loci were reported to be associated with clonal expansion in previous HIV integration studies. No significant hotspots of viral integration site were found. Our data revealed for the first time, that in both PBMC and rectal tissues, HIV integrated into the host genome as previous studies: 1) not clustered in the transcription start site; 2) prefer integrate into the gene regions (~60-75% in gene region); and 3) no substantial signs of clonal expansion has been observed.

665. A Serum-Free Method for Efficient Genetic Modification and High-Level Hemoglobin Production in Human CD34+ Cell-Derived Erythroid Cells

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In vitro erythroid differentiation from primary human cells is a valuable model for development of genetic strategies aimed at RBC diseases. However, erythroid differentiation methods are not optimal to evaluate hemoglobin (Hb) production following genetic modification. In this study, we sought to improve both genetic modification and Hb production in human CD34+ cell-derived erythroid cells with lentiviral transduction. We transduced human CD34+ cells with an EGFP-encoding lentiviral vector at MOI 50, and these cells were differentiated into erythroid cells using IMDM-based differentiation media containing 20% fetal bovine serum (FBS) and 2U/ml erythropoietin (EPO) (HEMA media, Cell Transplant. 2010). Two weeks later, we evaluated erythroid maturation (evidenced by high %GPA and low %CD71) and transduction efficiency (%EGFP) among GPA+ erythroid cells, and analyzed Hb production by Hb electrophoresis. To improve transduction specifically for the erythroid lineage, we transduced CD34+ cells at 1 or 2 days after initiating differentiation, and compared to transduction in serum-free X-VIVO10 media at 1 day before differentiation. Unexpectedly, lower %EGFP was observed after transduction during differentiation than transduction before differentiation ($p<0.01$). We hypothesized that lentiviral transduction is inhibited by FBS contained in differentiation media; therefore, we investigated StemSpan-based serum-free differentiation media (2U/ml EPO) with lentiviral transduction at 2 days after initiating differentiation. We observed higher %EGFP in the serum-free media than FBS-containing HEMA media ($p<0.01$), while the serum-free media resulted in insufficient erythroid differentiation with lower %GPA, higher %CD71 ($p<0.05$), and undetectable Hb production by electrophoresis. We hypothesized that additional nutrients (lipids and amino acids) are essential for efficient erythroid differentiation in serum-free media; thus, we added a lipid mixture into StemSpan-based serum-free erythroid differentiation media, or replaced 20%FBS with 20% knockout serum replacement (KSR; including lipid-rich albumin) in HEMA media (IMDM+KSR). Either lipid or KSR supplementation in serum-free differentiation with transduction resulted in higher %EGFP ($p<0.01$) and partially improved erythroid differentiation with comparable %GPA ($p<0.05$ and ns), higher %CD71 ($p<0.01$), and detectable Hb production, compared to HEMA media. Furthermore, we added 40% Ham's F12 nutrient mixture (F12; including various amino acids) into the HEMA-based serum-free differentiation media with 20%KSR (IMDM+KSR+F12), resulting in higher %EGFP ($p<0.01$) and equivalent erythroid differentiation with comparable %GPA ($p<0.01$), comparable %CD71 ($p<0.01$), and high-level adult Hb production detectable by electrophoresis, compared to FBS-containing HEMA media. In summary, we developed an efficient lentiviral transduction method for erythroid cells differentiated from human CD34+ cells using serum-free differentiation media. KSR and F12 supplementation allowed for high-efficiency transduction, robust erythroid differentiation, and high-level Hb production sufficient for analysis by Hb electrophoresis. Our *in vitro* erythroid differentiation system provides a practical evaluation platform for Hb production among human erythroid cells following genetic manipulation.

Differentiation media	EGFP	GPA	CD71	Hemoglobin
HEMA (IMDM + Serum)	20-69%	**80-97%**	**11-43%**	**++**
StemSpan (Serum-free)	**93%**	74%	66%	+/-
StemSpan + Lipid	**85%**	**93%**	**44%**	+
IMDM + KSR	**92%**	**96%**	**34%**	+
IMDM + KSR + F12	**90%**	**97%**	**32%**	**++**

666. High Resolution Longitudinal Monitoring of HSC Transplantation Using the NIS Reporter Gene and PET/CT Imaging

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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. Because each mammalian species expresses this endogenous protein in their thyroid, we can choose to use species specific NIS in the respective preclinical model through human clinical trials. Here, we used murine NIS as a reporter gene to monitor the transplantation and engraftment of lentiviral transduced primary murine hematopoietic stem cells in lethally irradiated mice. The murine NIS cDNA was driven by the SFFV promoter in a VSV.G pseudotyped HIV lentiviral vector. Our goal is to monitor specific clonal population of cells in vivo for as long as possible to better understand the relationship between time and clonal expansion in bone marrow repopulation. Mice were lethally irradiated at 900 cGy and received 10e5 to 10e6 NIS or luciferase expressing freshly isolated and lentiviral transduced unsorted or Sca+ HSC. Mice were imaged at regular intervals with bioluminescence imaging or PET-CT imaging with F18-tetrafluoroborate. Bioluminescence imaging is highly sensitive but lacks resolution. We were able to detect the Luc-HSC but were not able to discern the exact location of the cells. In contrast, PET imaging of NIS-HSC was highly productive, and we could easily discern individual foci of engrafted HSC within the bone marrow of the spine, pelvis, knees and the long bones. Signals were strong at 3 weeks post transplantation for both imaging modalities, and stabilized over 3-6 weeks. Using unsorted cells, the signals started to disappear by 6 weeks. In contrast, we could image Sca positive long term repopulating cells for longer. Interestingly, signals in NIS-HSC started to diffuse and localized foci of NIS expressing cells were no longer detected by 12 weeks post transplantation. Animals harvested at 16 weeks post transplantation indicated that NIS positive cells constituted about 15-20% of the regenerated cells in the bone marrow. We are currently performing deeper analysis of the imaging data to track individual foci of cells and to try to understand the reason for the reduction in NIS positive foci in the bone marrow despite the presence of NIS expressing cells in the animals.

Metabolic, Storage, Endocrine, Liver and Gastrointestinal Diseases

667. Correction of 21-Hydroxylase Deficiency (Congenital Adrenal Hyperplasia) by Systemic Administration of an AAVrh.10 Vector Coding for 21-Hydroxylase

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Congenital adrenal hyperplasia (CAH), the most common (>95%) inborn error of adrenal function and cause of adrenal insufficiency in the pediatric age group, is an autosomal recessive disorder occurring in 1:10,000-1:20,000 live births. CAH is the result of mutations in the *CYP21A2* gene, encoding the adrenal steroid 21-hydroxylase (21OH) enzyme. Cardinal phenotypic features of CAH include genital ambiguity, rapid postnatal growth and sexual precocity, and in severe cases, neonatal salt loss, failure to thrive, fatal hypovolemia, shock, and death. Current standard of care consists of life-long oral steroid replacement to reverse the cortisol deficiency, with chronic injections of glucocorticoids in rare instances of adrenal crisis. Although significant advances in the treatment of CAH have been made in recent decades, the burden and high costs of a life-long therapeutic intervention is not ideal for quality of life. We hypothesized that a one-time administration of an adeno-associated virus (AAV) gene transfer vector expressing the coding sequence of 21OH would provide persistent expression of the normal human 21OH cDNA in the adrenal glands to restore normal cortisol synthesis obviating the need for repeated corticosteroid administration. To evaluate adrenal transduction by AAV vectors, we tested two routes of administration, intra-adrenal (i.a.) *vs* the less invasive intravenous (i.v.) administration using the rh.10 serotype AAV expressing the luciferase reporter gene. Strikingly, 12 wk post administration both routes showed high expression of luciferase activity in the adrenal glands. We next evaluated the efficacy of the therapeutic gene in the AAVrh.10-21OH-HA vector ($6.5x10^{11}$ genome copies) by systemic administration (i.v.) into 7 wk old female 21-hydroxylase deficient (21OH-/-) mice, which have the CAH phenotype of adrenocortical hyperplasia and accumulation of progesterone, the aldosterone precursor. Immunohistological analysis 6 wk post vector administration, revealed expression of 21OH-HA in the zona fasciculata and the x-zone regions of the adrenal glands. By 2 wk post vector administration, serum levels of progesterone were dramatically reduced from 221 ng/ml (± 57 ng/ml) to 22 ng/ml (± 15 ng/ml; $p<0.008$ before treatment *vs* after treatment, $p>0.1$ treated *vs* wt control). The therapeutic efficacy remained persistent for at least 6 wk post administration, the longest time point evaluated to date, whereas untreated 21OH-/- mice continued to display high levels of progesterone. Additionally, ACTH levels decreased to 1014 pg/ml (± 138 pg/ml), similar to wild-type levels 602 pg/ml (± 101 pg/ml), 2 wk post administration while untreated mice had 2600 pg/ml (± 306 pg/ml, $p<0.008$ treated *vs* untreated, $p<0.03$ treated *vs* wt control). Taken together, these results demonstrate that a single treatment

with AAVrh.10-21OH-HA has the potential to provide a long term correction for deficiency of 21OH, representing a potential paradigm shift in current therapeutic approaches.

668. Transcriptional Targeting of Human Alpha and Beta Cells Using Recombinant AAV

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The decrease and eventual loss of beta cells and functional defects in glycemic control are hallmarks of the pathogenesis of both type 1 and type 2 diabetes. Direct genetic manipulation of pancreatic beta cells *in vivo* is a promising therapeutic strategy to induce immune tolerance and/or cellular regeneration, mitigate oxidative stress, and restore beta cell mass and function. Increasingly, alpha cells have been shown to be attractive targets for reprogramming to beta cells. Therefore, an efficacious *in vivo* gene therapy for diabetes will require specific targeting and transgene expression or genome editing of pancreatic endocrine cells. To address the lack of safe and useful human beta and alpha cell-specific vectors, we are developing transcriptionally targeted recombinant AAV (rAAV) that will specifically transduce pancreatic endocrine cells in human islet xenografts. We have created a rAAV mini-library of putative and known beta and alpha cell promoters and enhancer elements (NKX6-1, INS, PDX1, NEUROD1, GCG, GC, F10, FAP, ARX, TM4SF4, SMIM24). This 1st-generation rAAV mini-library is representative of 12 different promoters, 14 enhancers, and control CMV enhancer-minimal promoter constructs—distributed in multiple replicates among 164 clones, each with unique tandems of RNA barcodes. We used this rAAV mini-library of defined cis-regulatory elements (CRE) to transduce intact human islets *in vitro* for seven days. Preliminary deep sequencing results of FACS-sorted beta and alpha cells showed RNA barcodes that were highly represented in the two cell types with minimal overlaps. Of the top nineteen RNA barcodes expressed in cells (9 for alpha and 10 for beta), four RNA barcodes were shared, while the rest were unique to each cell type. These highly transcribed RNA barcodes corresponded to nine of the twenty-six CRE constructs. Four CREs were preferentially expressed in beta cells (INSx3 promoter and enhancers Pdx1.e1, E1R2f NKX6-1, and E1R1r NKX6-1), while only E1R1f NKX6-1 enhancer was preferred in alpha cells. Both alpha and beta cells activated the same five CREs (promoters F10, NEUROD1, and GC; enhancers Pdx1.e2r and NKXe1). These CREs produced between 10% and 60% of the expression levels of the CMV promoter control. Furthermore, we are also developing a next-generation rAAV-CRE library, which is made of compact gene regulatory elements that govern high levels of cell type specific expression in alpha or beta cells. These synthetic alpha or beta cell-specific cis-regulatory modules (CRMs) each consisted of unique combinations of short and unique transcription factor-binding motifs. As a proof-of-principle we have created a library of hundreds of CRMs composed of shuffled transcription factor binding motifs (MAFA, NEUROD1, PDX1, ISL1, E2A, USF, HEB, CREB,CEBPß) present in the human insulin promoter. Thirty of the CRMs we screened ranged in length between 200-800 bp and consisted of 6 to 18 motifs that will drive the expression of RNA barcodes. This strategy will enable us to simultaneously assess thousands of artificial CRMs in primary human islet xenografts. Eventually, beta/alpha-specific CRMs will be vectorized to encode transgenes for restoration of beta cell mass, immune tolerance, and function.

669. Haematopoietic Stem Cell Gene Therapy Combined with an Anti-Inflammatory to Correct Mucopolysaccharidosis IIIB

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Mucopolysaccharidosis Type IIIB (MPSIIIB) is an autosomal recessive lysosomal storage disease (LSD) caused by α-N-acetylglucosaminidase (NAGLU) deficiency, an enzyme involved in degradation of the glycosaminoglycan heparan sulfate (HS). This leads to accumulation of partially degraded HS in lysosomes and the extracellular matrix, giving rise to cellular dysfunction with devastating clinical consequences. Affected individuals exhibit severe central nervous system (CNS) degeneration with progressive cognitive impairment and behavioural problems, alongside more attenuated somatic symptoms. Enzyme replacement therapy is ineffective, as NAGLU enzyme cannot cross the blood brain barrier to where it is needed. In order to target the CNS, we have developed a haematopoietic stem cell gene therapy approach (HSCGT) in a mouse model of MPSIIIB, synthesising a high-titre lentiviral vector driving the expression of NAGLU under the control of the myeloid-specific CD11b promotor (LV.CD11b.NAGLU), and an IGFII peptide targeted approach (LV.CD11b.NAGLU-IGFII). Haematopoietic stem cells from MPSIIIB mice were transduced with LV.CD11b.NAGLU and transplanted into myeloablated 8 week-old MPSIIIB mice. At 8 months of age, mice were compared to wild-type, untreated MPSIIIB, MPSIIIB receiving a wild-type bone marrow transplant (WT-BMT) and mice treated with the steroid prednisolone (Pred) or combined Pred + LV.CD11b.NAGLU. NAGLU enzyme activity was substantially increased in the brain of LV.CD11b.NAGLU/IGFII treated mice, indicating effective delivery of NAGLU enzyme via monocyte trafficking and engraftment. This led to correction of the MPSIIIB behavioural phenotype in treated groups with normalisation of distance moved, average speed and frequency entering the centre in open field tests. In addition, we observed significant correction of astrogliosis and lysosomal compartment size in the brains of LV.CD11b.NAGLU treated mice, with normalisation of the expression of inflammatory cytokines TNFα, IL1B, IL1RN, MCP-1 and MIP-1α. HS levels were reduced to wild-type levels, with a concomitant reduction in HS sulphation. Interestingly Pred alone reduced peripheral inflammation and was able to correct behaviour phenotypes in MPSIIIB mice, despite no obvious reduction in astrogliosis. Although anti-inflammatory drugs cannot cure brain pathology they do relieve neurological symptoms. Synergistic effects in the periphery were also apparent when used in combination with LV.CD11b.NAGLU, highlighting the effectiveness of a combined strategy. We demonstrate neurological disease correction of MPSIIIB mice by HSCGT. Significantly, correction was superior to wild-type transplant alone, demonstrating proof of principle for the development of a clinical trial to improve neurological function in patients.

670. Tissue and Cell Type-Specific rAAV Gene Therapy Reveals a Novel Therapeutic Cell Target and Biomarker for Canavan Disease

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N-acetylaspartate (NAA) is one of the most abundant molecules in the mammalian central nervous system (CNS), with unknown function. Mutations in the aspartoacylase (ASPA) gene, responsible for hydrolysis of NAA presumably in oligodendrocytes, leads to Canavan Disease (CD) and NAA elevation in both brain and urine, serving as unique biomarkers. The current paradigm centers around oligodendrocytes for pathomechanism and treatment of CD. We hypothesize that ASPA restoration in non-oligodendrocyte cell types can rescue the CD phenotype and allow us to investigate the role of NAA in- and outside the CNS. To this end, we created rAAVs for tissue and cell type-specific hASPA expression by promoter restrictions to rapidly assess their disease modifying properties in astrocytes (As), oligodendrocytes (Oligo), neurons (Nr), liver (Lv), heart (Ht), and muscle (Ms), respectively, in short lived (4 weeks) CD knock-out (CD KO) mice. In terms of overall health as measured by body weight, motor function and survival, we found no difference between untreated KO and mice treated with rAAVHt.*hAspA* and rAAVMs.*hAspA*. When subjected to neuroimaging analysis, those animals also showed strong T2 hyperintensities on MRI and NAA elevation by MRS, well correlated with the characteristics of Canavan brain. Interestingly, rAAVLV.*hAspA* partially rescued KO mice from lethality without normalization of motor function or NAA levels. Although rAAVOligo.*hAspA* treatment indeed rescued CD mice from lethality, limited functional recovery was accomplished, possibly due to the poor transduction of oligodendrocytes by the rAAV9. We are currently testing an alternative capsid with possibly higher oligodendrocyte tropism. To our surprise, rAAV-mediated astrocytic expression of hASPA resulted in full rescue of CD mice from lethality and complete normalization of motor function, neuropathology and NAA metabolism. Beside therapeutic outcome assessment, neuronal specific expression of rAAVNr.*hAspA* and NAA quantification allowed us to investigate possible feedback mechanisms in NAA synthesis. Most interestingly, we found that low levels of hASPA expression from a weak neuronal promoter caused significant increase in NAA to levels beyond untreated KO mice, suggesting that partial restoration of NAA breakdown might have re-activated a positive feedback mechanism for NAA synthesis. In addition, we identified a new potential biomarker which shows reduction in serum after restricted hASPA expression in peripheral organs, suggesting possible roles of peripheral tissue in CD pathomechanism as well as potential benefits of treating peripheral tissues in CD gene therapy. Overall, our preliminary data present evidence that oligodendrocytes are not the only biologically significant cells in CD pathomechanism and gene therapy, and that a newly identified biomarker might allow us to further investigate the role of non-oligodendroglial cells in CD. Currently, we are exploring whether NAA levels in the periphery and CNS are independent of each other and what role the liver-brain-axis could entail. *These authors contributed equally

671. Correction of the Niemann-Pick Type C2 Mouse Model by Intracisternal Administration of an rh.10 Serotype AAV Vector Expressing the Npc2 cDNA

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Niemann-Pick type C2 (NPC2) disease is a rare, fatal neurodegenerative disorder caused by mutations in NPC2, leading to lysosome accumulation of unesterified cholesterol and other lipids. Clinically, NPC2 is characterized by organomegaly, liver dysfunction and severe neurological manifestations, resulting in early death. Similar to other lysosomal storage diseases affecting the central nervous system (CNS), there is no effective therapy for NPC2. We hypothesized that a one-time, intracisternal administration of an adeno-associated virus (AAV), serotype rh.10 gene transfer vector expressing the coding sequence of the mouse Npc2 (AAVrh.10-mNpc2-HA, HA tagged to facilitate analysis), would correct the neurologic deficit in Npc2 knockout mice (Npc2-/-). To assess the efficacy of this approach, we administered AAVrh.10-mNpc2-HA vector at 10^{11} genome copies (gc) to 6 wk old Npc2-/- mice (n=4M/5F) via the intracisternal route. These mice recapitulate characteristics of human NPC2 disease, including the hallmark CNS neurodegeneration and liver disease phenotype. Untreated Npc2-/- mice were sacrificed at 16 wk of age due to an advancing moribund state, while AAV treatment of Npc2-/- mice significantly extended lifespan. A subset of treated mice was sacrificed at 16 wk to compare with untreated littermates at the same age. Compared to untreated Npc2-/- and wild type mice controls, the histopathological findings in the AAV-treated Npc2-/- mice showed a significant amelioration of the disease pathology in the CNS and, interestingly, the liver, despite CNS administration of the vector. The cerebellum of AAVrh.10-mNpc2-HA treated mice showed markedly reduced storage of unesterified cholesterol aggregates as compared to age matched untreated Npc2-/- mice, and the disease hallmark of Purkinje cell loss in the treated mice was dramatically reduced. Additionally, CD68 staining, a marker protein for activated microglia/macrophages, was detected in the cerebellum of untreated but not the treated Npc2-/- mice, demonstrating the AAV treatment reduced disease-related CNS inflammation. As early as 2 wk post-administration of AAVrh.10-mNpc2-HA there was treatment-related reduction in serum disease markers (female > male): (1) low density lipoprotein (female, $p<0.05$ treated *vs* untreated, $p>0.4$ treated *vs* wt control; male, $p>0.9$ treated *vs* untreated, $p>0.2$ treated *vs* wt control); (2) alanine aminotransferase (female, $p<0.0006$ treated *vs* untreated, $p>0.9$ treated *vs* wt control; male, $p<0.002$ treated *vs* untreated, $p>0.08$ treated *vs* wt control); and (3) aspartate aminotransferase (female, $p<0.0003$ treated *vs* untreated, $p>0.05$ treated *vs* wt control; male, $p<0.0002$ treated *vs* untreated, $p<0.05$ treated *vs* wt control). Histopathological analysis of the liver was consistent with the biochemical results showing a reduction of lipid-laden macrophages in treated mice and amelioration of the liver phenotype. Taken together, these results demonstrate a benefit of a one-time intracisternal administration of AAVrh.10-mNpc2-HA with the potential for a life-long treatment of CNS and liver manifestations of NPC2.

672. Rescue and Phenotypic Correction in Argininosuccinate Lyase-Deficient Mice by AAV8.*ASL* Transfer

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Background: Argininosuccinate lyase (ASL) is central to two metabolic pathways: i) the urea cycle in the liver, which detoxifies the highly neurotoxic compound ammonia and ii) the citrulline-nitric oxide cycle, which synthesises nitric oxide from L-arginine. Patients deficient in ASL present with argininosuccinic aciduria (ASA), characterised by hyperammonaemic crises and a multi-organ disease with a severe neurological phenotype. Current therapeutic guidelines focus on normalising ammonaemia but not the systemic nitric oxide imbalance. Methods: We investigated the pathophysiology of the neurological disease in ASA using the knock-in $Asl^{Neo/Neo}$ mouse model. Neonatal or adult mice were administered a single-stranded AAV8 vector containing mouse ASL and the woodchuck hepatitis post-transcriptional regulatory element under the transcriptional control of the EFS promoter. Results: In mutant mice, we observed neuronal pathology associated with oxidative/nitrosative stress. AAV-treated mice showed long-term partial correction of both pathways: i) the urea cycle after a single intraperitoneal injection of vector in adult ASL-deficient mice ($2.5x10^{11}$vg/mouse); ii) the citrulline-NO cycle in the brain after a single intravenous injection at birth ($3.2x10^{11}$vg/mouse). Neuronal disease persisted after normalisation of only the ammonaemia but was dramatically reduced after correction of the neuronal ASL activity. Improvement of behavioural studies supported these results. Discussion: This demonstrates i) the key role of a neuronal disease independent from hyperammonaemia in ASA and ii) the potency of AAV to target the central nervous system and viscera, acting on two different metabolic pathways via a single vector. This provides new hope for neuro-hepatotropic inherited metabolic diseases.

673. AFP-Reporter Construct for Enhanced Human Induced Pluripotent Stem Cell-Derived Hepatocyte Isolation for Treatment of Arginase Deficiency

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Urea cycle disorders (UCDs) are incurable genetic diseases that disrupt the body's ability to metabolize ammonia into urea, leading to hyperammonemia. For arginase deficiency, a mutation in the final enzyme in the cycle, arginase 1 (A1), results in hyperargininemia, developmental delays and disabilities, seizures, and in serious cases, death. There is currently no completely effective treatment available. Advances in human induced pluripotent stem cell (hiPSC) research and genome-editing technologies have facilitated the genetic modification of stem cells for potential cellular replacement and gene therapies. In a recent study, we applied such technology to derive A1-expressing hepatocyte-like cells (HLCs) from patient-derived arginase-deficient hiPSCs [Mol Ther Nucleic Acids. 2016 Nov 29;5(11)]. However, though our group and others have derived HLCs demonstrating appropriate phenotypic and functional qualities, current HLC differentiation protocols suffer from inefficient derivation, line-to-line variation, and risk for lingering tumorigenic pluripotent stem cells. In this study, we aimed to address these limitations by developing a novel **A**lpha fetoprotein (AFP)-**R**eporter **C**onstruct (ARC) making capable the isolation of highly pure populations of AFP+ HLCs. Methods: Human embryonic stem cells and hiPSCs were derived and modified with the ARC for site-specific integration into the adeno-associated virus integration site 1 (AAVS1) safe harbor locus enabling FACS purification and blasticidin-mediated isolation of AFP+ hepatocyte derivatives. After confirming specificity of integration by Sanger sequencing, ARC-modified human pluripotent stem cells (hPSCs) were differentiated to HLCs and AFP+ cells were isolated via FACS and blasticidin treatment. Results: The ARC was developed to provide a pure population of hepatocyte-lineage cells by selecting AFP-expressing cells by encoding an AFP enhancer/promoter driving expression of tdTomato and blasticidin resistance. ARC function was first validated by fluorescence imaging of tdTomato after transfection into HepG2 cells. A transcription activator-like effector nuclease-based gene editing system was then utilized for in-frame integration of the ARC 3' of the AAVS1 gene in hPSCs. By targeting the 3' AAVS1, puromycin antibiotic resistance (PuroR) expression was linked by a self-cleaving 2A peptide ensuring only PuroR expression upon in-frame integration at the correct site. After ARC modification, purification, clonal expansion, and sequence confirmation, hPSCs were differentiated to HLCs and treated with blasticidin. tdTomato+ populations were quantified and isolated via FACS demonstrating 93.5% tdTomato+ HLCs compared to 57% tdTomato+ in HLC populations in which ARC selection was not used. Blasticidin-treated populations, assessed via qRT-PCR, exhibited higher expression of hepatic genes compared to ARC-unselected populations. Discussion: In this study, we demonstrated the ability of our ARC to purify an AFP+ population of hPSC-derived HLCs. With further optimization of blasticidin treatment, we expect to achieve higher purity of AFP+ HLCs post-differentiation. Also, to determine whether isolated AFP+ HLCs lead to better engraftment *in*

vivo, ongoing studies aim to transplant these cells into an established immunosuppressed liver repopulation mouse model. Though we have successfully demonstrated restoration of enzyme function in patient-specific hiPSCs and relevant cellular derivatives, the ability to isolate consistent and safe cellular populations for transplant into patients will be an invaluable tool in developing cell replacement therapies for treatment of UCDs and other single enzyme liver deficiencies.

674. Evaluation of Efficacy and Safety in a Dose-Escalating Nonclinical Study of a Clinical Candidate Vector in a Mouse Model of Crigler-Najjar

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Crigler-Najjar syndrome is an autosomal recessive disorder of bilirubin metabolism that occurs as a result of a partial or complete absence of uridine diphosphate glucuronosyl transferase 1A1 (UGT1A1) enzyme activity and is characterized by elevated bilirubin levels in the blood. UGT1 knockout (KO) mice, a model of Crigler-Najjar, display lethal hyperbilirubinemia in the immediate postnatal period. Phototherapy via exposure to blue florescent light for 12 hours per day from birth up to 21 days after birth allows UGT1 KO mice to survive until adulthood with serum total bilirubin levels of 9.1 ± 3.0 mg/dl. Pretreatment with phototherapy allows gene therapy vector administration to be delayed until after the most proliferative phase of liver development, which more closely simulates the likely clinical scenario. The purpose of this study was to determine the minimally effective dose (MED) of a clinical candidate vector in a mouse model of Crigler-Najjar. UGT1 KO mice 6-20 weeks of age received an intravenous injection of one of four doses of vector AAV8.TBG.hUGT1A1co ($2.5x10^{10}$, $2.5x10^{11}$, $2.5x10^{12}$, and $2.5x10^{13}$ genome copies [GC]/kg, n = 5/sex/group). A cohort of animals received vehicle only. Mice were bled biweekly throughout the study to evaluate serum total bilirubin levels and liver transaminases. Animals were sacrificed on day 56 and blood was collected at necropsy for serum chemistry and hematology panels. Sacrificed animals were necropsied and tissues harvested for a comprehensive histopathological examination. Efficacy of the vector was determined by serum total bilirubin levels, and Western blot and immunohistochemical analyses were performed to evaluate hUGT1A1 protein expression in liver. There were no apparent clinical sequelae in any groups, and abnormalities in clinical pathology were restricted to elevations in the liver transaminases, where ALT ranged from 1- to 9.1-fold over baseline levels. Transaminase abnormalities were primarily found in male mice at day 28 post vector administration of the highest dose of vector and were dose-dependent, with essentially no findings in animals that received lower doses of vector. Minimal to mild histopathological findings were present in the liver of male mice administered with the highest vector dose, including centrilobular single cell hepatocellular necrosis/degeneration, a finding which was also present in one male mouse administered with the vehicle control. At doses greater than $2.5x10^{10}$ GC/kg, there was a complete reversal of total bilirubin levels to baseline levels of 0.1-0.3 mg/dl. Administration of $2.5x10^{10}$ GC/kg resulted in a decrease in serum total bilirubin levels in male mice to 2.2 mg/dl (79% reduction) at day 14 post vector administration, which gradually increased to 4.3 mg/dl at day 28, and returned to baseline hyperbilirubinemia by day 42. Therefore, the MED for sustained and complete normalization of total bilirubin levels in a majority of animals in the mouse model of Crigler-Najjar is equal to $2.5x10^{11}$ GC/kg.

675. Combination of Gene and Enzyme Replacement Therapies for Mucopolysaccharidosis Type V

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Enzyme replacement therapy (ERT) is the standard of care for several lysosomal storage diseases. ERT, however, requires multiple and costly administrations and has limited efficacy. We recently showed that a single high dose [2x10e12 genome copies (gc)/kg] administration of adeno-associated viral vector serotype 8 (AAV2/8) is at least as effective as weekly ERT in a mouse model of mucopolysaccharidosis type VI (MPS VI). However, the administration of high doses of AAV2/8 requires a challenging and costly production process and might result in both cell-mediated immune responses and insertional mutagenesis. We therefore evaluated whether the combination of low doses of AAV2/8 with a less frequent ERT schedule (monthly) than canonical (weekly) may be as effective as the single treatments at high doses or frequent regimen. We found that levels of correction in mice receiving the combined therapy were similar to normal controls as previously observed in mice administered with single treatments at high dose of AAV2/8 or weekly schedule of ERT. Since no amelioration in skeletal dysplasia was observed in adult mice treated with either high dose of AAV2/8 or frequent regiment of ERT, we are currently testing if combining weekly neonatal ERT and administration of 2x10e12 gc/kg of AAV2/8 in adult mice is more effective than each single treatment at improving bone abnormalities. Finally, we will investigate whether further reduction of ERT frequency is as much effective when combined with a single administration of AAV2/8. In summary, our data show that low dose gene therapy can be successfully used as a means to reduce the frequency of ERT administration. Based on this, further combinations of gene and enzyme replacement therapies deserve to be investigated to improve the efficacy of treatment for MPSVI and concomitantly reduce both the risks and costs associated with either therapy.

676. AAV Gene Therapy for Phenylketonuria

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Phenylketonuria (PKU) is an autosomal recessive genetic disorder caused by the attenuation of phenylalanine-4-hydroxylase (PAH) activity, resulting in the buildup of phenylalanine in the tissues and

blood. High levels of phenylalanine in the bloodstream are thought to inhibit the transport of other large neutral amino acids across the blood brain barrier, affecting brain development and resulting in intellectual disability and seizures. Treatment for PKU is currently limited to maintenance of a strict phenylalanine-restricted diet and products directed at stabilizing residual PAH. We believe it is possible to improve upon the current standard of care with a liver-targeted AAV gene therapy approach. To investigate the development of gene therapy for PKU, we created four unique mouse strains by inducing different mutations in exon 1 of the *PAH* gene by CRISPR/Cas9 technology. We performed a natural history study on each of these strains to determine the progression of the disease and identify the strain that best replicated the human PKU phenotype. PKU colonies, designated B and C, both contain a single base pair (bp) deletion at different locations in exon 1 and maintain average phenylalanine levels of 2049 μM and 1705 μM, respectively, compared to normal levels of 70 μM. PKU colony A, despite having a 64 bp deletion and a 3 bp insertion in exon 1 of the *PAH* gene, has a modestly higher average phenylalanine level of 477 μM. PKU colony D, which has a 6 bp deletion, has phenylalanine levels equivalent to wild type littermates. Following AAV8 vector administration at a dose of 1E12 GC/kg for expression of a human codon optimized version of *PAH* to the PKU B mouse colony, plasma phenylalanine levels were reduced by 87% to 222 μM. This reduction in plasma phenylalanine levels restored the ability of the males to produce offspring. These results represent the first steps toward development of an AAV-based therapeutic for PKU.

677. Enhanced CNS Transduction in MPS IIIB Mice

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Mucopolysaccharidosis type IIIB (MPS IIIB) is an autosomal recessive lysosomal disease caused by defective production of the enzyme α-N-acetylglucosaminidase. It is characterized by severe and complex central nervous system (CNS) degeneration. Effective therapies will likely target early onset disease and overcome the blood-brain barrier. Modifications of adeno-associated viral (AAV) vector capsids that enhance transduction efficiency have been described in the retina. Herein, we describe for the first time, therapeutic assessment of two intracranially administered capsid mutated Adeno-associated virus serotype 8 variants, AAV8(double Y-F) and AAV8(double Y-F + T-V), for the treatment of MPS IIIB in a neonatal setting. We evaluated biodistribution and transduction profiles of both variants compared to the unmodified parental AAV8, and assessed whether the method of vector administration would modulate their utility. Vectors were administered through four intracranial routes: six sites (IC6), thalamic (T), intracerebroventricular (ICV) and ventral tegmental area (VTA) into neonatal mice. Overall, we conclude that the IC6 method resulted in the widest biodistribution within the brain. Noteworthy, we demonstrate that GFP intensity was significantly more robust with AAV8(double Y-F + T-V) compared to AAV8(double Y-F). This provides proof of concept for the enhanced utility of IC6 administration of the capsid modified AAV8(double Y-F + T-V) as a valid therapeutic approach for the treatment of MPS IIIB, with further implications for other monogenic diseases.

678. h*NAGLU* Codon-Optimization Enhances Secretion of rNAGLU, Leading to Functional Correction of MPS IIIB in Mice Following Systemic rAAV9-h*NAGLU*^{op} Delivery

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Mucopolysaccharidosis (MPS) IIIB is a lysosomal storage disease caused by autosomal recessive defect in α-N-acetylglucosaminidase (NAGLU), leading to lysosomal GAG accumulation in virtually all organs and profound neurological disorders and somatic manifestation. No treatment is currently available for MPS IIIB. Previously, we developed an effective systemic rAAV9-h*NAGLU* gene delivery approach for treating MPS IIIB (IND approved). In an attempt to improve the efficacy of AAV-h*NAGLU* gene delivery, we have developed a 2nd-generation AAV vector containing a codon-optimized h*NAGLU* cDNA driven by a CBA promoter. Our in vitro studies showed that the new AAV-CBA-h*NAGLU*^{op} vector mediated effective expression and significantly enhanced secretion of rNAGLU. Further, a single IV injection of rAAV9-CBA-h*NAGLU*^{op} vector in MPS IIIB mice at age 1m or 6m led to the rapid and persistent restoration of NAGLU activity and the clearance of lysosomal storage pathology throughout the CNS, peripheral nervous system (PNS) and broad peripheral tissues. Importantly, we demonstrate long-term neurological benefits with significant improvement in cognitive and motor function, and extension of survival (ongoing) in MPS IIIB mice treated at age 1m or 6m with an IV injection of 1e13vg/kg or 2e13vg/kg rAAV9-h*NAGLU*^{op} gene delivery. These data demonstrate the promising clinical potential of systemic rAAV9-h*NAGLU*^{op} gene delivery for treating MPS IIIB at both early and advanced disease stages.

679. Development of a Gene Therapy Program for Barth Syndrome

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Barth syndrome (BTHS) is caused by a single gene mutation in tafazzin (*TAZ*) that results in abnormal mitochondria, skeletal myopathy and heart failure. Currently there are no effective therapies for BTHS other than supportive cardiac care. Our program aims to: 1) identify relevant clinical and physiologic outcome variables, 2) identify the optimal AAV-*TAZ* expressing vector in a) BTHS mice and b) human induced pluripotent stem cell (iPSC)-derived cardiomyocytes and myotubes, 3) test the safety of AAV9 *TAZ* expressing vectors through primate toxicology studies and 4) test the efficacy and safety of AAV9 *TAZ* gene therapy in patients with BTHS. Aim 1: In 24 BTHS and 27 unaffected human subjects, we have identified impaired exercise tolerance (VO_{2peak}), lower cardiac strain (echocardiography), altered cardio-skeletal energetics (^{31}P magnetic resonance spectroscopy), and blunted

exercise fatty acid oxidation rate (stable isotope tracer methodology) in BTHS vs. controls. Aim 2: Our preliminary vector comparison data suggest that administration of $1x10^{13}$ vector genomes/kg of either dsAAV9-CMV-*TAZ* (ubiquitous distribution), dsAAV9-Taz-*TAZ* (*TAZ* natural distribution) *or* dsAAV9-Des-*TAZ* (desmin promoter, cardio-skeletal distribution) to neonates or adults corrects disease phenotypes in a BTHS mouse model (see abstract by S. Suzuki-Hatano et al.). Based upon these encouraging findings, we have collected BTHS human peripheral blood mononuclear cells and reprogrammed them into iPSCs. The BTHS iPSCs are being differentiated into cardiomyocytes and skeletal myotubes to characterize functions and enable further testing of the effectively of AAV-*TAZ* vectors in human cell types representing a variety of different mutations in the *TAZ* locus. Aims 3, 4: IND-enabling and human studies have been planned. Our current data have identified relevant cardio-skeletal muscle functional and physiologic outcomes in human subjects as well as suggested efficacy of the AAV9 expression vectors.

Neurologic Diseases (including Ophthalmic and Auditory Diseases) III

680. AAV9-Based Gene Therapy in Aspartylglucosaminuria Mice

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Aspartylglucosaminuria (AGU) is an autosomal recessively inherited lysosomal storage disease. It is caused by the absence of functional lysosomal enzyme aspartylglucosaminidase (AGA), resulting in the accumulation of AGA substrate, aspartylglucosamine (GlcNAc-Asn) in different body fluids and tissues. In humans, AGU is a progressive disorder characterized by intellectual disability, skeletal abnormalities, and early mortality. Currently, there is no cure for AGU and patient care usually includes medical, social, and educational rehabilitation. Due to the failure of bone marrow transplantation and the lack of feasibility of enzyme replacement therapy in this disease, gene therapy becomes a key strategy which might provide a therapeutic benefit. Supporting this, previous adenovirus-mediated gene therapy was demonstrated to be effective in locally reducing lysosomal storage in the brain (intraparenchymal route) and fully correcting it in liver (IV route) of AGU mice. Over the past decade, adeno-associated virus (AAV) gene therapy has progressed rapidly and many groups have shown that AAV9 vectors can confer a dramatic therapeutic improvement to various neurological disorders. Therefore, we hypothesized that AAV9-based gene therapy may impart a therapeutic benefit to AGU mice. To test this hypothesis, a construct carrying the codon-optimized human AGA gene was packaged into AAV9 capsids as a self-complementary (sc) genome. The scAAV9/AGA vectors were then injected by tail vein into adult AGU mice at $2x10^{11}$ vg/mouse. Mouse serum and urine were collected to measure serum AGA activity and urine GlcNAc-Asn excretion, respectively, at one week before and multiple time points post injection. Our results clearly show that IV injection of scAAV9/AGA vectors dramatically increases serum AGA activity 1 week post injection to a super physiological level (treated KO vs KO vs Het mice: 736.4±46.9 vs 0 vs 10.0±1.3 nmol/24hr/ml serum). By 4 weeks post injection, serum AGA activity in about half of treated mice is sustained. In the other half, activity decreases to less than one third of that at 1 week post injection but is still significantly higher than in heterozygous mice. There is no further decrease of serum AGA activity in any of those treated mice between 4 to 32 weeks post injection. On the contrary, urine GlcNAc-Asn excretion decreases substantially in scAAV9/AGA vector treated groups at 4 (treated KO vs KO vs Het mice: 13±14 vs 1132±473 vs 0 mg GlcNAc-Asn/g creatinine) and 8 (treated KO vs KO vs Het mice: 14±14 vs 786±429 vs 0 mg GlcNAc-Asn/g creatinine) weeks post injection. Taken together, these results suggest that IV administration of scAAV9/AGA vectors can almost completely clear peripheral GlcNAc-Asn accumulation in AGU mice. Similar results have been achieved in mice dosed by lumbar intrathecal injection. Various behavioral tests, small animal imaging, and histopathological staining are being used to determine if these treatment benefits extend to the central nervous system. The results from the study suggest that gene therapy could be considered for possible future human translation.

681. Gene Therapeutic Restoration of Peripheral Olfactory Impairment in BBS1 Knockout Mouse Model of Bardet-Biedl Syndrome

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Olfactory loss and dysfunctions are pervasive but underappreciated health concerns that affect personal safety, ingestive behavior, social interactions, and overall quality-of-life. Patients with olfactory impairments have limited therapeutic options, particularly those associated with congenital diseases. Bardet-Biedl Syndrome (BBS) is one such disorder with an incidence of 1 in 100,000 births. BBS patients exhibit olfactory impairments and other symptoms resulting from defective cilia morphology and/or function in various cell types/tissues throughout the body. Despite having a relatively healthy olfactory epithelium, olfactory sensory neurons (OSNs) of BBS mutant mice models lack the ability to build/maintain cilia, rendering the neurons incapable of odor detection. Here, we examined OSN ciliary defects in *Bbs1* mutant mice, whose genetic mutation account for approximately 30% of all BBS patient cases. Utilizing *Bbs1* mutant mice, we assessed the utility of gene replacement therapy to restore ciliation and function in both young and adult mice. *Bbs1* mutant mice possessed fewer and shorter OSN cilia in which BBSome protein trafficking was defective. In addition, we observed loss of acute odor-evoked responses, diminished glomerular sizes, and decreased tyrosine hydroxylase immunostaining.

Intranasal administration of wild-type *Bbs1* gene in adenovirus restored OSN ciliation, corrected BBSome cilia trafficking defects, and returned acute odor responses. As a step toward developing a clinically relevant therapy for gene-associated olfactory impairments, we assessed the capacity of adeno-associated viral infection of the olfactory epithelium and demonstrated for the first time the ability of AAV9-mediated delivery of wild-type *Bbs1* to restore ciliation and odor detection in *Bbs1* mutants. Together, our data demonstrate that OSN ciliogenesis can be promoted in differentiated cells of young and adult *Bbs1* mutants, and highlights the potential of gene replacement therapy as a viable restorative treatment for congenital olfactory disorders.

682. Optical Coherence Tomography Profiles, Visual Acuity and Ranibizumab Usage During 3-Year Follow-Up in a rAAV.sFLT-1 Gene Therapy Clinical Trial for Wet Age-Related Macular Degeneration

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Purpose: Optical coherence tomography (OCT) is widely used in follow-up and management of wet age-related macular degeneration (wAMD) patients. Here we present the OCT profiles and best corrected visual acuity (BCVA) in wAMD patients injected with rAAV.sFLT-1 as well as the number of *pro re nata* ranibizumab (RBZ) retreatments during 3-year follow-up in a Phase I clinical trial (ClinicalTrials.gov; NCT01494805). **Methods** Eligible patients ≥65 years with wAMD and BCVA 3/60—6/24 in the study eye and 6/60 or better in the other eye were randomised to treatment (n=6) and control (n=2). Treatment patients received 100μL of low dose (10^{10} vg, n=3) or high dose (10^{11} vg, n=3) rAAV.sFLT-1 via subretinal injection and were analysed in a single group called "treatment group" (TG). All patients were monitored monthly to the primary endpoint at 12 months, then at the discretion of the treating physician through month 36; protocol-driven study visits during this period after 12 months at 18 and 36 months. Eyes were monitored with a spectral domain-OCT device and by BCVA as part of the protocol. All patients received intravitreal RBZ at baseline, 1 month, and at subsequent visits for evidence of active wAMD. **Results** Two TG patients withdrew from the study after month 18 and do not have 36-month data. There was a reduction in median centre point thickness (CPT) of 61.0 and 103.5 μM from BL at 18 and 36 months, respectively. BCVA at baseline was 40.0 (IQR: 33.0-54.0) Early Treatment for Diabetic Retinopathy Study (ETDRS) letters. At 18 months, there was an increase in median BCVA of 6.5 ETDRS letters from baseline, and a loss of 1.5 ETDRS letters from baseline at 36 months. Of the four patients who had assessments at 36 months, each showed findings consistent with advanced wet AMD at baseline and at 36 months. Three of the four patients showed no appreciable progression of disease activity relative to the 12-month examination. The fourth patient developed vitreomacular traction associated with an intraretinal cyst. These four patients received a median of 3.0 (IQR: 1.5-4.3) RBZ retreatments through 36 months of the study. **Conclusions** In this small cohort, OCT and BCVA findings, and the low median number of RBZ retreatments, suggests that gene therapy warrants further evaluation as a potential treatment option for wAMD.

683. 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. The most common form of GM1 gangliosidosis affects children, is fatal by 4 years of age, and is characterized by rapidly progressing and fatal neurological disease. Outside of palliative and supportive care, there is no effective treatment for GM1. AAV, or adeno-associated viral 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 thalami and deep cerebellar nuclei. However, this injection route is invasive therefore intravenous delivery was studied to circumvent the risk of damage while potentially to increasing cortical and systemic biodistrubution. AAV9 was delivered at a total dose of 1.5e13 vector genomes/kg body weight at approximately 1 month of age. The six animals included in the study were divided into two cohorts: 1) a long term group, which will be allowed to go to humane endpoint, and 2) a 16-week post treatment group. After the designated time point, biodistrubution of enzyme and vector was assessed. The long term group (n=2) currently shows a 4.5-fold increase in life expectancy, with both animals showing limited clinical signs. The short term cohort (n=4), also showed amelioration of clinical symptoms prior to collection. Across the cerebrum there was an average 0.3 fold normal β-galactosidase (β-gal) activity as well as an average of 0.6 fold normal β-gal activity in the cerebellum in the short term treated cats. Additionally, there was a reduction in the lysosomal enzymes mannosidase, hexosaminidase A and hexosaminidase total, which are elevated in untreated animals. Additional methods to determine the efficacy of IV administration of AAV gene therapy include magnetic resonance imaging, MR spectroscopy, aspartate aminotransferase and lactate dehydrogenase analysis in the cerebrospinal fluid, and vector biodistrubution via qPCR. Taken together, this data suggests that IV injection of AAV gene therapy may be a safe and effective treatment of GM1 gangliosidosis.

684. AAV Delivery of Cell Penetrating Proteins for Treatment of Retinal Disease

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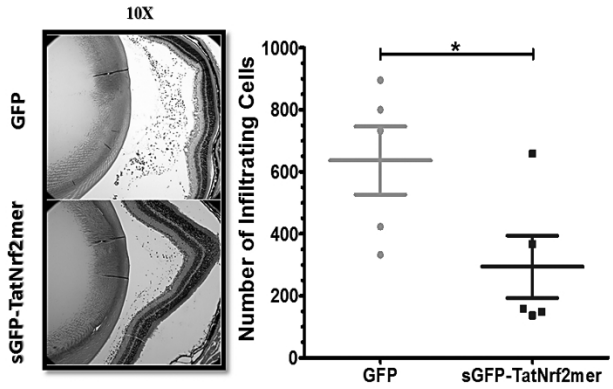
Gene therapy for retinal disease typically employs subretinal injection in order to access photoreceptor cells and the retinal pigment epithelium (RPE). This is a complicated surgical procedure that is

performed in a hospital setting. In addition, subretinal injections detach the retina from the RPE, and detachment may not resolve in the case of a diseased or aged retina. We have been developing gene therapies for inflammatory diseases of the retina that are designed to suppress the activation of the NLRP3 inflammasome or to activate the antioxidant response regulated by the transcription factor Nrf2. To deliver therapeutic proteins or peptides to the photoreceptors and RPE, we designed an AAV vector to deliver secreted, cell penetrating proteins or peptides. Therapeutic cargoes include an Nrf2 derived peptide to block the interaction between Keap1 and Nrf2, a caspase activation and recruitment domain (CARD) peptide to suppress the processing of Caspase 1 by the inflammasome, and the M013 protein of Myxoma virus, which also blocks inflammation. AAV serotype 2 with five capsid modifications (4 Y to F; 1 T to V) was used to deliver genes for the secreted proteins to the vitreous compartment in mouse models of acute oxidative stress ($NaOI_3$ injection) and an of ocular inflammation (uveitis). This method of treatment was effective in blocking inflammatory infiltrates and in permitting recovery from acute oxidative injury to the RPE. These vectors are currently being tested in a mouse model geographic atrophy, the advanced dry form of age related macular degeneration. The AAV-Tat CARD vector appears to stabilize photoreceptor function in this model, suggesting that it may be beneficial in preventing progression of this blinding disease.



685. Early AAV9 Mediated Repletion of the Glucose Transporter 1 Protein Improves Brain Microvasculature Defects and Glut1 Deficiency Syndrome

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Abstract Glut1 deficiency syndrome (Glut1 DS) is a severely disabling neurodevelopmental disorder caused by haploinsufficiency of the *SLC2A1* gene and thus reduced levels of its translated product, the Glucose Transporter Type 1 (Glut1) protein. Although one consequence of insufficient Glut1 protein - a paucity of brain glucose - is widely recognized, precisely how this condition results in the complex Glut1 DS phenotype is unclear. Here we demonstrate that reduced Glut1 protein has a profoundly deleterious effect on brain angiogenesis and on the maintenance of the cerebral microvasculature, without compromising the blood-brain barrier. Early, AAV9-mediated repletion of the protein in neonatal Glut1 DS model mice ensures the proper development of the microvasculature of the brain and arrests disease evolution. Augmenting the protein in juvenile mutant mice that have already suffered sustained low brain glucose is less effective in establishing normal brain microvasculature, yet reverses mutant brain glucose concentrations and has a pronounced overall therapeutic effect. In contrast, delayed delivery of the protein - to adult mice - neither reverses defects of the brain capillary network nor rescues the Glut1 DS phenotype. Our results link brain dysfunction in Glut1 DS to novel defects of the cerebral microvasculature, a condition that likely upsets the evolving circuitry of the maturing brain. The results, furthermore, identify a limited postnatal therapeutic window for Glut1 DS, yet suggest that timely reinstatement of the Glut1 protein, even under prolonged conditions of profoundly low brain glucose, can prevent disease. Early repletion of the Glut1 protein may thus constitute the most effective treatment yet for Glut1 DS.

686. Assessment and Optimization of DNA/RNA Barcode Sequencing for Comparing Transduction Capabilities of Numerous Adeno-Associated Virus (AAV) Strains Following Intravitreal Injection

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Recent success in adeno-associated virus (AAV) mediated gene therapy correction of inherited retinal diseases has excited the field. Initial clinical studies utilized subretinal injections of AAV vectors, an approach with significant drawbacks due to the invasiveness of the injection. An alternative approach relies on the injection of vectors directly into the vitreous fluid allowing a wider-dissemination of viral vectors throughout the eye with minimal damage to the retina. While this strategy is appealing, the inner-limiting membrane (ILM) of the retina significantly retards the ability for AAV vectors to transduce outer retinal cells of interest. Efforts to overcome the restriction have identified a number of mutated AAV capsids that display increased levels of outer retina transduction following intravitreal injections, although no comprehensive data exists that compares all of these mutants to each other. The AAV DNA/RNA barcoding technique previously developed by our lab is an effective approach to collect such data and help establish the most effective vectors. In this approach, each AAV capsid strain is packaged with a unique vector genome that contains a known pair of 12 nucleotide barcodes under the control of the human U6 snRNA promoter for expression of RNA barcodes after cell transduction. Retinas are harvested post-mortem and homogenized, from which the RNA and DNA is extracted for the downstream processing required for Illumina sequencing. Due to this, maximizing the recovery of quality RNA and DNA is of great desire. To address this, we compared the recovery of DNA and RNA from neural retina's of C57BL/6J mice using Trizol and Qiagen's Allprep kit, finding that on average Trizol provided nearly twice as much total DNA as the Allprep kit (approximately 6 µg vs 3 µg from one retina), as well as substantially more RNA (approximately 6 µg vs 0.1 µg from one

retina), although these yields were lower than expected based on the total number of cells in the retina. In order to evaluate if there was any potential that the reduced quantity of RNA and DNA may bias or skew the barcode sequencing data we are analyzing the effects on findings when a single retina is used for analysis versus those found when 2 or 4 retinas are combined, providing insight into the diversity of DNA/RNA recovered, as well as the minimum amount of input DNA/RNA required for this approach. Once optimized, this technology can be used to obtain meaningful data regarding the efficiency of numerous AAV strains to transduce the cells of the retina following intravitreal injections. To this end, we designed a side-by-side comparison experiment in which we produced a DNA/RNA-barcoded library containing AAV serotypes 1-11, rh8, rh10, common mutants including AAVDJ, 2i8, 2G9, and LK03, as well as all published AAV2, 5, 8, and 9 tyrosine-to-phenylalanine and threonine-to-valine mutants, AAV2-7m8, AAV6shH10, and Anc80. We also included the following three mutants, AAV9 PHP.A, PHP.B, and G2A12, that displayed the ability to cross the blood-retinal barrier but were not tested in intravitreal injections. The data obtained from this library will provide important data allowing us to rank the abilities of different AAV strains to transduce retinal cells after intravitreal injections, providing beneficial information to the field to help choose vectors best suited for use in the eye. This system and approach is amenable for studies in animal models from rodents to non-human primates, which will allow us to compile transduction data to identify if a difference exists in the transduction capabilities of AAV strains between species.

687. Nonviral Delivery of BDNF mRNA Polyplex Improves Recovery After Spinal Cord Injury in Mice

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Recovery after Spinal Cord Injury (**SCI**) is inhibited by inflammation and apoptosis in the days after the initial injury[1]. These secondary processes not only inhibit regeneration in the injured site, but can kill cells in the surrounding healthy tissue. Scar formation in the injured site also prevents neuron regrowth into the site. It is imperative that treatments are developed to prevent these processes and promote neuron regeneration after SCI to help improve quality of life.

Brain Derived Neurotrophic Factor (**BDNF**) is a secreted protein that has been shown to improve outcome in SCI animal models. BDNF binds to TrkB receptor on neurons and activates MEK-ERK and PLCγ1 pathways, promoting cell survival and synapse plasticity. BDNF protein delivery has been investigated in a variety of CNS diseases with promising results in animal models, but difficulties involved in protein delivery have limited BDNF's use in humans[2].

Gene delivery could overcome these issues by delivering DNA or RNA encoding BDNF, causing cells to overexpress the protein at the site of injury. A previous study[3] prepared BDNF plasmid DNA polyplexes using a biocompatible cationic polymer developed in our lab. Mice were intrathecally injected with 2 μg of polyplex, and then given contusion SCI at 24 hours post injection. Mice that received BDNF DNA showed better motor function recovery than mice that received no DNA or luciferase DNA.

However, DNA-based BDNF delivery carries potential problems of its own. Long term exposure to high concentrations of BDNF has been associated with adverse effects, including weight loss and Schwann cell proliferation, and uncontrolled expression of growth factors is potentially carcinogenic. Additionally, plasmid DNA has a small chance of randomly inserting into the genome, potentially damaging endogenous genes and regulatory networks. DNA delivery is also limited by nuclear entry, which makes transfection of non-dividing neurons difficult. mRNA delivery might overcome these issues because it does not need to enter the nucleus. It can transfect a high percentage of dividing and non-dividing cells, and produce large amounts of protein. mRNA will not integrate into the genome, and degrades, limiting the expression of potentially dangerous proteins.

In this study, BDNF mRNA polyplexes were made with a PEGylated version of the polymer from the previous DNA study. Mice were given contusion SCI using a precision impactor device, then immediately injected with a single dose of 500 ng of mRNA polyplex directly into the injured spinal cord tissue. Mice were observed for 2 - 6 weeks following injury, and motor function recovery was monitored using Basso Mouse Scale (**BMS**) scoring and the CatWalk gait analysis instrument.

Mice that received BDNF mRNA showed greater motor function improvement than injured mice who did not receive mRNA. Improvement was detected using both the subjective BMS system and the more objective automated CatWalk system. BDNF mRNA mice showed significantly better measurements in several parameters as early as 2 weeks post-injury, which persisted for at least 6 weeks. CatWalk analysis showed enhanced improvement in contact area, swing speed, stride length, step regularity, and other parameters. These results show that early treatment with BDNF mRNA polyplex can significantly improve recovery after traumatic spinal cord injury by reducing neuron death during the secondary injury, and might improve quality of life in human patients if dosed soon after injury.

References

1. J Neurotrauma. 17, 915-26. Oct 2000.

2. Nat Rev Drug Discov. 10, 209-19. Mar 2011.

3. J Control Release. 197, 1-9. Jan 2015.

688. Gene Therapy Correction of Frataxin Deficiency in a Novel Mouse Model of Friedrich's Ataxia

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Friedriech's ataxia (FRDA) is an autosomal recessive disorder that primarily affect the nervous system and the heart. It affects 1 in 50,000 individuals in the United States. It is caused by mutation in the frataxin (FXN) gene (intronic expansion of GAA triplets). The resulting FXN protein deficiency leads to inefficient mitochondrial electron transfer, iron accumulation, and oxidative damage. 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 for FRDA disease progression is available. The purpose of this ongoing preclinical investigation is to correct FXN deficiency in the central nervous system and the heart by delivering an AAV9 vector that expresses human FXN. Our hypothesis is that intravenous (IV) delivery of rAAV9-CBA-FXN will restore the bioavailability of cellular FXN and prevent the consequences of FXN deficiency in the heart and nervous system. In this study, we used a novel FRDA mouse model based on FXN gene silencing via shRNA knockdown induced by doxycycline. The model presents more uniform nervous system and cardiac manifestations than previously described murine models of FRDA. Our preliminary results showed that IV injection with AAV9-CBA-FXN prevents weight loss and death. The untreated group showed a significant decline in weight ($p<0.05$; mean weight loss ≈30%) and increased mortality (3/5 animals died). In contrast, all the animals in the vector treated groups (10/10) were rescued. Based on these observations, we are currently conducting a larger study to determine the effect of AAV9-CBA-FXN induced restoration of FXN levels on both cardiac and the neurologic disease phenotypes. In order to characterize these changes a large array of functional, behavioral, physiological and imaging outcome measures is being used. The neurologic outcomes include grip strength, open field, rotarod, sensory evoked potential and MRI/MRS. The cardiac outcomes included EKG, echocardiogram and MRI. 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.

689. Intraganglionic Gene Delivery of the Photosensitive Chloride Channel, IC++, Facilitates Transdermal Light-Mediated Inhibition of Neuropathic Pain in Rodents

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Optogenetics has been established as a powerful tool to study the central and peripheral nervous systems with its potential for directly treating human disease tantalizingly on the horizon. Optogenetic inhibition of pain in mice has been shown to be effective and provides an attractive initial application. We aim to translate this approach to treat neuropathic pain in humans. The novel blue-light activated chloride ion channel, iC++, was packaged into AAV serotype 6 and tested in the chronic constriction injury (CCI) mouse model of neuropathic pain. The AAV was delivered by nerve injection after the injury and onset of pain to better replicate the clinical time course. Two weeks after injection, we observed that transdermal delivery of blue light could inhibit mechanical allodynia, presumably through hyperpolarization of nerve endings by an intracellular chloride flux. Notably, the observed pain inhibition was more robust for iC++ than observed with the yellow-light activated chloride pump, NpHR. We next tested a novel surgical approach to deliver the iC++. Transforaminal epidural injection is a common clinical procedure used to deliver steroids adjacent to the dorsal root ganglia (DRG) for treating disc herniation pain. The approach has recently been modified to deliver AAV directly to the DRG in large animals and therefore provides an attractive method to restrict gene therapy to the target cells in neuropathic pain. Intraganglionic injections of AAV5 expressing iC++ were made at L4 and L5 spinal levels in rats with preexisting neuropathic pain. Three weeks after vector delivery, we observed transdermal light mediated inhibition of mechanical and thermal allodynia in two different models of neuropathic pain i.e. the tibia fracture/cast immobilization model of Complex Regional Pain Syndrome (CRPS) and the CCI model. Functional pain inhibition was observed up to 12 weeks at which point animals were sacrificed for histology. Interestingly, cell type characterization revealed transduction of multiple sensory neuron types with a trend for higher numbers of iC++ expression in large NF200 positive cells, that have been implicated in mediating mechanical allodynia. Taken together, our results confirm optogenetic therapy for neuropathic pain as a potentially attractive clinical application of this technology.

690. Enhancement of Normal Vascularization and Reduction of Retinal Vaso-Obstruction by Introducing Adeno-Associated Virus Expressing mTOR siRNA on a Rat Model of Oxygen Induced Retinopathy

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Purpose The purpose of this study is to investigate therapeutic effects of down-regulating mammalian target of rapamycin (mTOR) by a recombinant adeno-associated virus (rAAV) expressing mTOR small-interfering RNA (siRNA) on the retinal ischemia. Methods In vitro analysis, adult retinal pigment epithelial (ARPE) 19 cells were transfected with mTOR or non-specific control siRNA under either normal or hypoxic condition. Real-time reverse transcription-polymerase chain reaction and immunoblot analysis were performed to detect the expression of mTOR, hypoxia-inducible factor 1α (HIF-1α), vascular endothelial growth factor (VEGF). For vivo delivery of siRNA, rAAV2 expressing corresponding mTOR or control siRNA with green fluorescent protein (GFP) were prepared. As an animal model, oxygen induced retinopathy (OIR) was prepared using rat pups. The rats were divided into three groups (n=5 per each group): 1. Vehicle-treated sham, 2. rAAV-siCon, 3. rAAV-simTOR. A total of 5 μL of intravitreal injection was administered for group 1, 2, and 3 at postnatal day 12 (P12). At P17, flat mounts of the retina were prepared for fluorescein angiography. Analysis of retinopathy in OIR was graded by automatic threshold method using ImageJ software. The severity of retinopathy was quantified by using the ratio of total pixel counts of retinal vascular area over the total retinal area. Results In vitro, both mTOR mRNA and protein levels decreased dramatically after mTOR siRNA treatment, whereas the control siRNA in ARPE 19 cells showed no change. In vivo experiments, signs of retinal vascular incompetence

such as retinal vascular dilation, tortuosity, vascular obliteration, and neovascularization were readily noticed in all of the subjects. Retinal angiogram of rAAV-simTOR group, however, indicated a significant reduction of avascular area in the retina in comparison to group 1 and 2 ($p<0.05$ and $p<0.005$, respectively), including the periphery and the central retina. MTOR siRNA significantly reduced both mTOR mRNA and protein levels in hypoxic condition, as well as in normal condition. In addition, HIF-1α protein and VEGF mRNA level significantly increased during hypoxia. Moreover, the mTOR siRNA treatment abolished the induction effect in HIF-1α protein by hypoxia, subsequently decreasing VEGF mRNA level. Conclusions Administrating rAAV expressing mTOR siRNA in the rat OIR model has beneficial effects improving vaso-obstructive pathologic conditions, possibly by reducing VEGF gene expression. This suggests the inactivation of mTOR pathway by mTOR siRNA may become an important novel therapeutic strategy in the treatment of various retinal vascular diseases.

691. Gene Therapy of AB-Variant GM2 Gangliosidoses in a Mouse Model Using Adeno-Associated Virus Serotype 9

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GM2 gangliosidoses are a group of neurodegenerative diseases affecting the brain. In humans, they are characterized by rapid neurological deterioration and death before 4-years of age. GM2 ganglioside is normally degraded in a cell's lysosomes through the action of three gene products, *HEXA*, *HEXB*, and *GM2A*. A defect in any one of these genes can result in a deficiency of Hexosaminidase A (HexA) enzyme activity toward GM2-ganglioside, which then cannot breakdown. The most rare form of these diseases, the AB-variant, is characterized by a mutation in the *GM2A* gene that encodes the GM2-activator protein, which is a required co-factor for the breakdown of GM2 gangliosides by the HexA protein. Currently, there is no cure for this disease. GM2A deficient mouse models provide an animal model to study potential therapies for the GM2 gangliosidosis. An effective viral vector known as Adeno-associated virus serotype 9 (AAV9) has previously been successful in treating the other two forms of GM2 gangliosidosis in preclinical trails. The aim of this study is to give a one-time treatment of AAV9.*GM2A* viral vector therapy at a dose of 1×10^{14} vector genomes per kilogram of mouse. Treatments were given to 1-day old pups via the superficial temporal vein, as well as adult mice via tail vein, to correct GM2-gangliosidoses AB variant. These mice are to undergo monthly behavioural testing, as well as biochemical and molecular analysis to be performed at 20 and 60-week end-points. We hypothesize that an optimized AAV9.*GM2A* treatment can correct the gene deficiency as well as phenotype of GM2-gangliosidosis AB variant in mice. Preliminary behavioural data does not show any statistical significance, which is what we expected since the phenotypic characteristics in the GM2A deficient mouse model develop after 20 weeks of age. Preliminary biochemical data for the short-term cohort, 20 weeks, showed a decrease in GM2 gangliosides, however not significant, of the treated mice when compared to vehicle treated. The development of such an improved approach for GM2-gangliosidosis will provide a step forward towards our goal of human clinical gene therapy trials.

692. Evaluation of AAV2tYF-GRK1-RPGR Vectors in a Canine Model of *RPGR*-XLRP

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Purpose: In previous studies, an AAV2tYF capsid and GRK1 promoter was found to be more efficient than an AAV5 capsid and IRBP promoter for transducing primate photoreceptors. As well, two stable human RPGR cDNAs (the codon-optimized RPGRco or shortened RPGRsk) were both effective at early-stage disease in the XLPRA2 dog model of X-linked retinitis pigmentosa caused by a mutation in *RPGR* exonORF15. We now compared the efficacy of 3 dose levels of RPGRco and RPGRsk vectors with a GRK1 promoter and AAV2tYF capsid delivered to XLPRA2 dogs at mid-stage disease (~12 wks of age; ~ 40 % loss of photoreceptors). **Methods:** Two dogs per group received a 0.15 mL subretinal injection of AAV2tYF-GRK1-RPGRco in the right eye and AAV2tYF-GRK1-RPGRsk in the left eye at each of 3 dose levels (1.2×10^{11}, 6×10^{11} or 3×10^{12} vg/ml). One dog received the mid-dose of AAV5GRK1RPGRco in both eyes and 1 dog received vehicle in both eyes. Rescue of photoreceptor structure was assessed by clinical examination, and histology/IHC on retinal cryosections at termination 8 weeks post injection. **Results:** No abnormal ophthalmic findings were noted in any eyes at the mid- or low-dose levels, but there was retinal inflammation and retinal detachment in the four eyes injected with the high-dose of either vector. Retinal perivascular infiltration consisting primarily in T cytotoxic (CD8) and B (CD20) lymphocytes, with some T helper (CD4) cells in the bleb area of all eyes injected with the high doses was also seen in one eye injected with AAV2tYF-GRK1-RPGRco at mid-dose. The AAV2tYF-GRK1-RPGRco and AAV2tYF-GRK1-RPGRsk vectors achieved dose-dependent RPGR transgene expression in canine photoreceptors, with greater RPGR expression in eyes injected with AAV2tYF-GRK1-RPGRco than in contralateral eyes injected with AAV2tYF-GRK1-RPGRsk at the same dose levels. Correction of rod opsin and middle/long wavelength (M/L) cone opsin mislocalization was demonstrated in all AAV-RPGR treated eyes. Müller cell activation was decreased in eyes injected with the low and mid-doses but not in eyes injected with the high dose, likely due to the associated inflammation and retinal detachment. **Conclusions:** With the AAVt2YF capsid and GRK1 promoter, greater RPGR expression was achieved with the vector expressing the RPGRco cDNA than with the vector expressing the RPGRsk cDNA. Optimal correction at mid-stage disease with limited inflammation, including improved structure of cones, correction of rod and M/L cone opsin mislocalization and reduced reactivity of Müller cells, was achieved in this model with the mid-doses of both vectors (0.15 mL at 6×10^{11} vg/ml).

693. Expanding the Utility of AAV to Treat "Large Gene" Retinal Diseases

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The safety and utility of recombinant adeno-associated virus (AAV) has been demonstrated in many preclinical contexts including ongoing clinical trials for retinal diseases. However, size constraints imposed by the packaging capacity of the vector restrict the breadth of therapeutic efficacy to diseases with coding sequence lengths that fit within the 4,700 nucleotide capacity of AAV. Though there are more than 250 identified genes with mutations associated with retinal disease, the most prevalent diseases with monogenic inheritance are caused by mutations in genes that exceed this capacity. Therefore, developing alternative therapeutic strategies that can utilize the biology of AAV while accommodating these "large genes" is highly desirable. We have designed an AAV that incorporates the technology of spliceosome mediated pre-mRNA *trans*-splicing (SMaRT), and we are currently investigating its efficacy as a therapeutic option to rescue expression of *CEP290*, which is a large gene that causes Leber's Congenital Amaurosis Type 10 (LCA10). We initially screened for and identified a binding domain to target this therapy to a central region of *CEP290* pre-mRNA. We are now testing the efficiency of this molecule by treating LCA10 patient-derived induced pluripotent stem cells, which can be used as a personalized model for rescuing expression of *CEP290*. These cells demonstrate inclusion of cryptic Exon X of *CEP290* due to a mutation in intron 26 (IVS26 c.2991+1655 A>G). This mutation creates aberrant splicing leading to an early stop codon, and it is present in a majority of LCA10 patients. Additionally, LCA10 patients with IVS26 are almost always compound heterozygous for a second CEP290 mutation. We are comparing our SMaRT LCA10 therapy in cell lines that will allow for comparison of rescue across many mutations within the *CEP290* gene.

694. The Role of Ectopic Striatal Nurr1 Expression on LID Development in the Parkinsonian Rat

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Levodopa (L-DOPA) is the leading pharmacological treatment for the motor symptoms in Parkinson's disease (PD). Unfortunately, chronic treatment with L-DOPA inevitably leads to the development of L-DOPA induced dyskinesias (LIDs), a series of involuntary motor behaviors including chorea, dystonia, and limb hyperkinesia. Much is still unclear about the molecular mechanisms leading to LIDs. Our group and others have shown that an ectopic induction of the transcription factor Nurr1 in both direct and indirect pathway striatal medium spiny neurons (MSN) is associated with LIDs. Nurr1, while a required factor for the dopaminergic neurons of the substantia nigra which degenerate in PD, is not endogenously expressed in striatal MSNs. Preliminary data from our lab has shown that LID severity can be affected by modulating Nurr1 expression with recombinant adeno-associated virus (rAAV). These data show that Nurr1 overexpression in the striatum plays an important role in LID development. The goal of the current studies is to determine if Nurr1 expression in the striatum—a region where Nurr1 is not normally expressed—can effect striatal physiology in a way that facilitates LIDs by changing 1) electrophysiological activity of the striatum, and/or 2) morphology of striatal MSN. 6-hydroxydopamine (6-OHDA) lesioned Sprague-Dawley rats received either rAAV-Nurr1 or rAAV-GFP injections in the striatum. Animals were not treated with L-DOPA and thus did not become dyskinetic. In one study, the local field potential (LFP) of the striatum following cortical stimulation was measured following a single dose of L-DOPA. While GFP-treated animals showed a depression in response, Nurr1-treated animals showed a potentiation. Remarkably, the response recorded in Nurr1-treated animals mimicked striatal LFPs recorded in established dyskinetic rats not treated with rAAV. In a separate study, rAAV-Nurr1 treated animals—with no L-DOPA treatment—show a decrease in thin-type spine density on MSNs, suggesting Nurr1 contributes to the loss of spines observed in direct pathway neurons. These studies that Nurr1 can induce a LID-like striatal physiology through changes in striatal activity and plasticity independent of L-DOPA. Ongoing studies are investigating Nurr1 in LIDs using D1 and D2-specific agonists to elucidate pathway-specific effects of Nurr1. Preliminary data suggests that ectopic Nurr1 expression can be induced in dyskinetic animals treated with D1-specific agonists, suggesting that D1 activation alone is sufficient for this abnormal expression. Together, these data implicate Nurr1 as a crucial factor in the molecular development of LIDs which needs to be fully understood as numerous studies from other groups aim to increase Nurr1 expression as a disease-altering treatment for Parkinson's disease.

695. Why Are scAAV9 Vectors Able to Pass Through the BBB but Not ssAAV9? ; The Difference Between the ssAAV9 and scAAV9 Vector in Transduction of CNS by Intravenous Injection

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Background: Adeno-associated virus (AAV) vector is a promising tool for gene delivery to the central nervous system (CNS) and is used for various neurological disorders, including Parkinson's disease, amyotrophic lateral sclerosis, and lysosomal storage disorders. However, the blood-brain barrier (BBB) is a key obstacle to gene delivery to the CNS by intravenous injection. We have previously reported that the single-stranded (ss) AAV vectors are able to pass through the BBB for at least 2 weeks after birth, but all serotypes of ssAAV vectors lose the ability to cross the BBB within 6 weeks (Miyake N. *et al.,* Brain Res. 2011). When we injected the ssAAV9 or self-complementary (sc) AAV9 vectors encoding EGFP into the tail vein of the 8-week-old mice and assessed EGFP expression immunohistochemically, minimal expression was detected in the mice administered with ssAAV9,

whereas efficient EGFP expression was achieved throughout the entire brain with scAAV9 transduction. These results raised a question why are scAAV9 vectors able to pass through the BBB but not ssAAV9? To address this issue, we analyze the difference between the ssAAV9 and scAAV9 vector in transduction of the CNS by intravenous injection. **Methods:** To analyze whether ssAAV9 or scAAV9 vector effectively pass through the BBB, we used *in vitro* BBB model system RBE-12 BBB Kit (Pharma Co-Cell Co. Ltd., Nagasaki, Japan). This system uses three cell types, such as primary cultures of rat (Wistar rat) brain capillary endothelial cells, brain pericytes and astrocytes, and the triple co-culture systems to show *in vivo* BBB features. We generated ssAAV9 and scAAV9 vectors encoding EGFP, and then transduced the BBB model according to the manufacture's protocol. Three days after the transduction, we estimated the transduction efficiency and copy number in the transduced cells. We also injected the ssAAV9 or scAAV9 vectors into the tail vein of the 8-week-old mice and analyzed the copy number in the CNS 1week after vector injection. **Results:** Transduction efficiency of scAAV9 for upper well (endothelial) cell was 5 to 10-fold higher than that of ssAAV9 (52% vs. 6%). qPCR analysis showed that similar copy number was detected in the scAAV9 or ssAAV9 transduced endothelial cells (29708 vs. 33896 copies/ng). When we analyze the transduction efficiency of astrocytes, we did not detect the transduced cells after ssAAV9 transduction. On the other hand, efficient transduction was observed with scAAV9 transduction. Interestingly, when we estimated the copy number of transduced cells, we did not find any differences between ssAAV9 and scAAV9 transduced astrocytes (6583 vs. 10570 copies/ng). *In vivo* experiments showed that there is so significant difference in the copy number in the CNS between the mice injected by the ssAAV9 and scAAV9 vectors (5.3 vs. 6.0 copies/ng). **Conclusion:** These results indicated that the both ssAAV9 and scAAV9 vectors are able to pass through the BBB and transduced the CNS cells in a similar kinetics. We speculate that the difference of expression in astrocytes and in vivo experiment may depend on the characteristics of scAAV, which is more stable than that of ssAAV.

696. 3D-Imaging and Tissue Reconstruction of Deep-Brain Gene Silencing with Nanoscale, Non-Viral siRNA Complexes

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Utilising nanomatrials for an effective and non-invasive in vivo nucleic acid delivery holds great promise for the development of modern therapeutics. Recent advances in nanomaterial-mediated effective siRNA transport leading to therapeutic gene silencing at localised brain foci following stereotactic administration are very promising. Here, we report nanoconstructs comprising amino-functionalised multi-walled carbon nanotubes (f-CNTs) or positively charged liposomes complexed with Bcl-2 targeting siRNA (siBcl-2) stereotactically injected into the striatum of C57BL/6J mice. The aim was to assess its potential as a targeted neuronal therapy to replace surgical resection in focal neurodegenerative diseases. Nanomaterial transport can increase cellular internalisation and residence time of siRNA into a diverse group of neuronal cells when compared to siRNA alone. Following deep-brain gene silencing of the pro-survival Bcl-2 was correlated with localised cellular apoptosis at the site of injection by TUNEL staining. To comparatively assess the spatial distribution of the induced apoptotic effect by both the nanotube- and liposome-based vectors, 3D imaging and reconstruction of the TUNEL-positive apoptotic region was developed. We are proposing the design and application of nanoscale, non-viral vectors as efficacious and targeted vectors for siRNA-mediated gene silencing deeply and focally in the CNS. Compared to surgical resection, this technology and approach could provide safer and less aggressive methods to achieve ablation of neuro-pathological structures.

Synthetic/Molecular Conjugates and Physical Methods for Delivery of Gene Therapeutics II

697. Extracellular Vesicles as Gene Therapy Vehicles for Duchenne Muscular Dystrophy

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Objective: Determine the efficiency and targeting potential of extracellular vesicles (EVs) for gene transfer to dystrophic skeletal muscle and heart. Background: Restrictions to the current AAV vector platform for Duchenne muscular dystrophy (DMD) gene therapy include pre-existing antibodies against the AAV capsid and dystrophin transgene product, T-cell mediated destruction of cells expressing the transgene and AAV capsid proteins, and poor muscle targeting after IV delivery. The use of muscle-specific promoters can reduce the risk of immunogenicity from AAV transgene expression. Incorporation of AAV into extracellular vesicles (EVs) ("vexosomes") requires no direct capsid modifications as it uses the endogenous properties of EVs for retargeting, while the EV itself acts as a shield for AAV-specific antibodies.

Methods: CK8e was prepared as a 436 bp regulatory cassette by combining key elements of the enhancer and promoter regions of the muscle creatine kinase gene and designed to drive high transgene expression that is restricted to skeletal muscle and heart. We used standard AAV9 and vexosome AAV9 to express GFP, firefly luciferase, and ΔR4-23/ΔCT micro-dystrophin (Harper, et al., 2002) in wild-type or mdx mice. Standard AAV9 was prepared by conventional means. Vexosome AAV9 was purified from conditioned media of 293T cells by gradient ultracentrifugation (Maguire, et al., 2012). We delivered identical doses of either standard AAV9 or vexosome AAV9 vectors by intramuscular or tail vein injections, and measured gene expression by serial in vivo bioluminescence imaging, in vivo fluorescence imaging, fluorescence microscopy of tissue sections, immunofluorescence, and Western blotting.

Results: In vivo bioluminescence imaging showed expression of vexosome AAV9-CK8e-luciferase was 1.5 - 4-fold higher in gluteal and hindlimb muscles, and identical in heart, as standard AAV9. Differential luciferase expression was evident as early as 1 - 2 weeks after

injection, peaked 4 - 6 weeks after IV injection, and remained largely stable for at least 8 months. Expression of GFP was similar in hindlimb, paraspinal, and heart of vexosome-AAV9 and standard AAV9-treated mice by serial in vivo imaging and Western blot. Immunofluorescence confirmed expression of vexosome AAV9 micro-dystrophin at the sarcolemma throughout treated mdx muscles 5 weeks after injection.

Conclusions: Vexosome AAV9 is a powerful gene delivery vehicle that performs as well or better than standard AAV9 in mouse skeletal muscle and heart. The differential luciferase expression evident within 1 - 2 weeks suggests a gene delivery advantage of vexosome over standard AAV9. CK8e is a candidate regulatory cassette to restrict AAV-mediated gene expression to muscle tissue in vivo. AAV9 and the CK8e regulatory cassette appear well suited for long-term expression in skeletal muscle and heart. Our results support further development of AAV9 vexosomes as gene therapy vehicles to help mitigate pre-existing immunity in DMD.

698. A Novel Peptide to Deliver Proteins and CRISPR Ribonucleoprotein Complexes in Therapeutic Cells

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Viral and non-viral delivery methods that introduce foreign genetic material in cells are widely used in cell therapy applications. While effective in certain cell types, current techniques are often detrimental to cell viability and cell potency, leave genetic footprints, and pose important safety and regulatory concerns. One promising avenue to bypass the use of foreign DNA and virus in cell therapy consists on the direct delivery of active proteins. However, the poor efficiency of current protein delivery methods, mainly caused by endosomal entrapment, slowed down the transfer of this approach toward cell therapy.

Aiming to develop an efficient protein delivery method fully applicable for human cell therapy, we designed proprietary peptides, named "Feldan Shuttles", that enable intracellular entry of native proteins without endosomal entrapment. Exempt from chemical modifications, the Shuttle and cargo are both degraded after their transient active use, a characteristic that decreases the regulatory burden associated with human cell therapy. Initially based on natural sequences, these carrier peptides that combine a cell penetration function with an endosomal leakage activity are now rationally designed and optimized for their ability to deliver proteins in mammalian cells by a simple co-incubation while preserving viability.

Using this peptide-based technology, we delivered a green fluorescent protein tagged with a nuclear localization signal (GFP-NLS) in several cell lines both in adherent (e.g. HeLa, GFP=81%, viability=95%), and suspension cultures (e.g. Thp1, GFP=82%, viability=95%). GFP-NLS delivery was also achieved in human primary cells, namely hematopoietic (GFP=60%, viability=95%) and mesenchymal (GFP=45%, viability=95%) stem cells as well as human myoblasts (GFP=24%, viability=69%) and NK cells (GFP=41%, viability=96%). As proof of concept of the therapeutic potential of the platform, we successfully delivered active intracellular proteins like the transcription factor HoxB4, small shark variable new antigen receptors, multiple monoclonal mouse antibodies and CRISPR ribonucleoprotein complexes containing spCas9 or asCpf1 nucleases. Most importantly, the native proteins delivered keep their intrinsic activity, as we demonstrated the binding of antibodies with their respective intracellular target and achieved genome edition using nucleases in multiple cell models including hard-to-modify human NK cells (Insertion/deletion = 45%) and myoblasts (Insertion/deletion = 12%). In the context of the optimization of CRISPR activity for therapeutic use, we successfully delivered multiples guide RNAs in a single experiment and used both Cas9 and Cpf1 simultaneously in NK cells.

This simple but powerful peptide-based technology opens new avenues in cell therapy like the use of antibodies with intracellular targets ("intrabodies") and allows modification of hard-to-transfect cells with high therapeutic potential like NK cells. Based on the ex vivo results that we have generated, the Feldan Shuttle could be a game-changer for the delivery of many other types of proteins for therapeutic applications.

699. Intra-Amniotic Delivery of Biodegradable Microparticles and Nanoparticles to the Spinal Defect in a Rat Myelomeningocele Model

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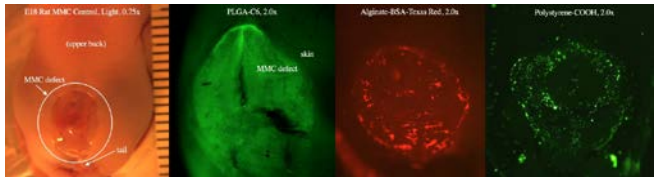
Background: Myelomeningocele (MMC), or open spina bifida, is a devastating spinal birth defect that results in lifelong neurological morbidity. Current treatments include high-risk prenatal surgery or postnatal soft tissue reconstruction after spinal cord damage has already occurred. We aim to develop a minimally invasive MMC treatment that delivers therapeutic agents to the MMC defect in utero and protects the spinal cord from injury. The specific aims of this study were to determine the feasibility of this new minimally invasive approach and to analyze the binding characteristics of various nano- and microparticles after intra-amniotic injection in a rat model of MMC. **Methods**: Time-dated pregnant rats were gavage fed all-trans retinoic acid on day 10 of gestation to induce MMC. The control group received no further intervention. On day 18, groups 2, 3, 4 and 5 underwent laparotomy and intra-amniotic (IA) injections of various particles. Group 2 received poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) loaded with coumarin-6 (C6) fluorescent dye. Group 3 received alginate microparticles (MPs) loaded with bovine serum albumin (BSA) and Texas Red® dye. Group 4 received green fluorescent polystyrene MPs modified with terminal carboxyl groups (COOH), and Group 5 received unmodified green fluorescent polystyrene MPs. All groups were sacrificed 3 hours after injections, and fetuses were examined under a fluorescence stereomicroscope. Images were analyzed with the Fiji image processing program. MMC defect-to-skin mean brightness ratios (MBR) were calculated using data from image overlays of equal area and analyzed by ANOVA with Tukey multiple comparisons test. **Results**: All dams (N=6) and fetuses

(n=57) were viable at the time of sacrifice. PLGA NPs (p=0.0461), alginate MPs (p<0.0001), and polystyrene-COOH MPs (p<0.0001) had significantly higher defect-to-skin brightness ratios compared to controls. The best MMC defect binding was achieved by polystyrene-COOH MPs (MBR=2.139, 95% confidence interval 1.768-2.510), followed by alginate MPs (MBR=1.957, 95% CI 1.44-2.48) and PLGA NPs (MBR =1.61, 95% CI 1.34-1.88).



Unmodified polystyrene MPs had the weakest (p=0.9796) and least specific binding (MBR=1.18, 95% CI 1.07-1.30) compared to the non-injected controls (MBR=1.09, 95% CI 1.013-1.168).

Conclusions: Intra-amniotic delivery of biodegradable alginate and PLGA particles to the MMC defect is feasible and does not cause immediate complications in rats. Polystyrene-COOH MPs, alginate MPs and PLGA NPs showed preferential binding to the MMC defect compared to surrounding skin. Specific binding to the MMC defect might be related to negative zeta potential or terminal functional groups. Preferential binding of biodegradable MPs and NPs to the MMC defect will allow for targeted delivery of encapsulated therapeutic agents in future studies.

700. Versatile Retargeting Lentiviral System Bearing an Isopeptide Bond Pair SpyTag-SpyCatcher

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Introduction: Selectivity/specificity is a crucial factor for the safety and efficacy of gene therapy vectors. It remains a non-trivial task to produce gene therapy vectors programmed to deliver genetic payloads efficiently and specifically to disease cells of interest. We sought to develop a facile strategy to reprogram lentiviral vectors through *in vitro* covalent functionalization with cell-binding proteins (e.g. monoclonal antibodies). **Methods:** An isopeptide bond-forming protein-protein pair is exploited to covalently functionalize a lentivirus with a cell-binding protein (CBP). This protein pair consists of the N-terminus (SpyCatcher) and C-terminus (SpyTag) of the collagen adhesion domain (CnaB2) from the fibronectin binding protein (FbaB) in *Streptococcus pyogenes*. SpyTag is genetically inserted into an exposed extracellular loop of the envelope protein of a binding-deficient fusion-competent Sindbis virus to form Sind-SpyTag, and the SpyCatcher protein is genetically or chemically linked to a CBP to form SpyCatcher-CBP. Coincubation of Sind-SpyTag-pseudotyped lentivirus (Sind-SpyTag-pp) with SpyCatcher-CBP triggers the covalent functionalization of the lentivirus with the CBP through an isopeptide bond and reprograms the lentivirus to cells displaying the binding partner of the CBP. **Results:** A HER2-binding DARPin (H-DARPin) and a transtuzumab-derived Fab (T-Fab) were used as model CBPs. SpyCatcher was genetically and chemically linked to DARPin and Fab, respectively, and loaded onto Sind-SpyTag-pp. The resulting H-DARPin- or T-Fab-functionalized Sind-SpyTag-pp were able to efficiently transduce HER2$^+$ cells with >10^6 IU/mL, while the naked Sind-SpyTag-pp exhibited minimum transduction in the same cells (<10^4 IU/mL). The association of CBP with Sind-SpyTag-pp was found to be irreversible due to the high stability of the isopeptide bond and the CBP-functionalized virions were able to selectively transduce HER2$_+$ cells in mixed cell population. Finally, Sind-SpyTag-pp is able to efficiently transduce HER2$^+$ cells in the presence of pooled human serum, suggesting a high potential for *in vivo* application in human gene therapy. **Conclusions:** We developed an *in vitro* chemical biological approach for covalent conjugation of a cell-binding protein to lentiviruses, providing a convenient and effective new tool for reprogramming lentiviral vectors to deliver their genetic cargo to specific cell types. Ongoing work in the lab aims to extend this strategy to functionalize lentiviruses with additional Fabs and assess the ability of the reprogrammed lentiviral vectors to deliver functional genetic cargo to desired cell types *in vivo*.

701. Tissue Repair by VEGF and HGF Gene Therapy Is Mediated by Cardiac Stem Cell Mobilization, Induction of Angiogenesis and Inflammation Control

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Chronic heart failure (HF) remains a crucial problem in modern societies. Despite many advances in management of HF, treatment options for many patients are limited and new strategies are required. Under these circumstances gene therapy got into scope for non-option or severe cases of HF to postpone transplantation and alleviate patients' status. The goal of our study was to assess the therapeutic potential of combined HGF and VEGF gene therapy for stimulation of angiogenesis and endogenous mechanisms of cardiac repair after myocardial infarction (MI). As a proof-of concept we evaluated VEGF, HGF or combined HGF+VEGF plasmid gene delivery in a rat model of MI. We found that within 14 days after injection of VEGF+HGF combination results were clearly superior to sole VEGF or HGF in terms of vascular density increase (capillary and arteriole), but was unable to significantly reduce post-MI fibrosis, yet certain trend was observed. HGF and HGF+VEGF injections significantly stimulated accumulation of c-kit+ resident cardiac stem cells in border zone and WT1+ epicardial cell activation when compared to VEGF plasmid injections. We have also found a reduction of inflammatory infiltration in border zone of MI in VEGF+HGF animals compared to VEGF group indicating decrement of inflammatory response in acute phase of MI. Further *in vitro* studies showed that production of pro-inflammatory MCP-1 and IL-6 by human endothelial cells was increased by VEGF and reduced by HGF. Combination of both factors resulted in medium levels of MCP-1 and IL-6 production, thus HGF seems to counteract

the pro-inflammatory action of VEGF. As for another chemokine - IL-8 both factors had stimulating effect on its production and their combination yielded maximum amount of IL-8 secretion by endothelial cells, which suggests an alternative way for IL-8 regulation involved. We can conclude that combination of VEGF and HGF has good translational promise for post-MI HF and cardiac regeneration. Deeper in-sights into their mechanism of action might show non-canonical or pleiotropic properties of VEGF+HGF combination and their role in tissue regeneration.

702. The Life of mRNA Vaccines in Dendritic Cells

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Messenger RNAs (mRNAs) are on the path of becoming a new class of drugs allowing vaccination against cancer, bacterial and virus infections. Although immune responses are generated by naked mRNAs encoding tumor antigens, their complexation with lipid-based formulations (LBFs) are designed to boost their specificity and internalization in dendritic cells (DCs) for better immune responses and dose reduction. To date, no study has tracked the complete cellular pathway of a protein-coding mammalian mRNA in an *in vivo* cell system at both spatial and temporal levels. Here we report a tracking study of mRNA formulated with lipid-based formulations cellular uptake to protein expression in the murine DC cell line (DC2.4 cells). We incorporated a 24 stable repeat of MS2 motif allowing single molecule imaging of mRNA in live cells in order to understand better delivery, routing and stability of mRNA leading to high transfection efficiency. Our study confirms that efficient delivery and mastering of intracellular fate of mRNA vaccines are the key for their success and translation to the clinic.

703. Cell-Specific Targeting Increases Intracellular Uptake of Nanoparticles in Cystic Fibrosis Bronchial Epithelial Cells

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Introduction: Cystic fibrosis (CF) is one of the most common lethal genetic diseases of childhood. Despite advances in supportive therapies, it is associated with significant multi-organ morbidity. The underlying cause of CF is an autosomal recessive mutation in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. Much of the severity of this disease is due to the absence of normal CFTR in airway epithelium resulting in abnormal luminal mucus and chronic lung disease. Gene therapy aimed at replacing or repairing the CFTR gene remains elusive. We recently demonstrated successful gene editing of the CFTR gene *in vivo* after nasal administration in adult mice using oligonucleotide-based editing reagents encapsulated in degradable nanoparticles (NPs). The aim of this study was to test the hypothesis that conjugation of cell surface-specific antibodies to these NPs would enhance their uptake in target cells and tissues. To this end, we 1) characterized several surface antigens of cystic fibrosis bronchial epithelial (CFBE) cells; 2) conjugated antibodies to these antigens to NPs; and 3) compared the uptake of cell-specific antibody-conjugated and isotype-conjugated NPs in CFBE cells.

Methods: Surface expression of the bronchial epithelial markers podoplanin (PDPN) and mucin 1 (MUC1) was determined in CFBE cells, a transformed, immortalized cell line homozygous for the disease-causing deltaF508 mutation in CFTR. Antigen accessibility, receptor density, and antigen-antibody affinity were determined by immunofluorescence and flow cytometry. Co-block poly(lactic acid)-poly(ethylene glycol) (PLA-PEG) NPs loaded with fluorescent dye were conjugated to bronchial epithelial-specific antibodies by carboxyl-amine crosslinking. CFBE cells were treated with NPs for two hours at three concentrations: 25 ug/ml, 50 ug/ml, 100 ug/ml. Uptake of antibody-conjugated NPs by CFBE cells was assessed by flow cytometry and compared with isotype-conjugated controls.

Results: The bronchial epithelial markers were localized to the CFBE cell surface by immunofluorescence. We were able to approximate receptor density and antibody affinity properties of each antibody and target pair. Conjugation of anti-MUC1 antibodies to NPs resulted in a significant increase in uptake by CFBE cells compared with isotype-conjugated NPs at 50 ug/ml ($p<0.05$) and 100 ug/ml ($p<0.0001$), showing 2.3- and 3.3-fold increased uptake, respectively. In contrast, PDPN-conjugated NPs did not show increased uptake above control, consistent with poor antibody-antigen affinity.

Conclusions: Internalization of NPs by CFBE cells is significantly enhanced by conjugation to anti-MUC1 antibody specific to the cell surface of CFBE cells. The ability to create targeting NPs specific to tissue types creates potential for enhanced delivery of gene editing reagents to CFBE cells *in vitro* and to affected tissues in CF, which will be further explored using a mouse model of CF.

704. Towards a Non-Viral Gene Delivery Platform That Emulates the Mechanism Used to Deliver the Adenoviral Genome

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Introduction: *in vivo* gene therapy relies on efficient delivery of DNA into the nucleus. Although viral vectors deliver DNA payloads efficiently, they suffer from drawbacks including limited payload size and immunogenicity. Alternative non-viral platforms lack intracellular and nuclear trafficking capabilities. Previous attempts using covalently attached nuclear localization signals failed to successfully deliver a large enough fragment of DNA[1]. A more efficient system is required,

ideally assembled using relatively simple production steps. Adenovirus (Adv) genomes have a single copy of terminal protein (TP) covalently coupled to the 5' end of each DNA strand via its Ser580. TP is known to involved in Adv replication and transcription[2] but its role in viral gene delivery across the nuclear pore is completely unexplored.

Aims: 1) Investigate the role of Adv TP in the delivery of the Adv genome. 2) Construct synthetic TP-DNA conjugates and evaluate their potential to improve the efficiency of non-viral gene delivery.

Methods: TP was mutated to replace serine 580 with cysteine. We used cysteine 580 for downstream chemical conjugations. TP was produced in BL21 E.coli as a thioredoxin fusion protein (TP-TRx) with 6-His tag, then purified using a HisTrap column (GE Healthcare) with *in situ* refolding on column. The TP fusion protein was tested using an Alphascreen assay (PerkinElmer) to probe for interactions with nuclear importins. Subsequently TP was labelled using maleimide-Alexa Fluor 594 and microinjected into the cytoplasm of HeLa cells (Injectman, Eppendorf). Maleimide-activated oligonucleotides were prepared using an amine-modified oligonucleotide and Sulfo-SMCC conjugated at a 1:40 molar ratio. Activated oligonucleotide was further conjugated to TP using different molar ratios.

Results: TP-TRx was expressed from a pET32a construct in bacteria grown in terrific broth (TB), and induced using 1mM IPTG. TP was accumulated as an insoluble fraction. After successful solubilisation with GuHCl, a refolding assay revealed successful refolding at pH > 8.5. The yield of TP-TRx was ~5mg of protein per L of TB medium. Refolded protein labelled with Alexa Fluor readily accumulated in the nucleus within 5 min of cytoplasmic microinjection, validated by co-injection of a cytoplasmic marker. TP interacted strongly with importin β or αβ complex with K_d of 21.17 or 58.83 nM, respectively. Our results suggest that TP does not interact with importin α alone. TP in both cleaved or non-cleaved form (TP-TRx) can be successfully conjugated to DNA oligonucleotides using OligoMaleimide:TP-cysteine conjugation ratios of 2:1 or above.

Conclusion: These results demonstrate that TP is functional after expression in bacteria and refolding. In addition, the TP can be conjugated directly to oligonucleotides for further ligating to a linear DNA payload. Current and future works focus on studying the delivery kinetics of TP-conjugated DNA using microinjection and digitonin permeabilization assays before progressing the system towards *in vivo* testing.

Bibliography: 1. Ludtke, J.J. et al. *J. Cell Sci.* **112,** 2033-2041 (1999).

2. Schaack, J. et al. *Genes Dev.* **4,** 1197-1208 (1990).

705. Antitumor Effect and Safety of Systemically Delivered Oncolytic Adenovirus Complexd with EGFR-Targeted PAMAM Dendrimer

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Adenovirus (Ad)-mediated cancer gene therapy has been proposed as a promising alternative to conventional therapy for cancer. However, success of systemically administered naked Ad has been limited due to the immunogenicity of Ad and the induction of hepatotoxicity caused by Ad's native tropism. In this study, we synthesized an epidermal growth factor receptor (EGFR)-specific therapeutic antibody (ErbB)-conjugated and PEGylated poly(amidoamine) (PAMAM) dendrimer (PPE) for complexation with Ad. Transduction of Ad was inhibited by complexation with PEGylated PAMAM (PP) dendrimer due to steric hindrance. However, PPE-complexed Ad selectively internalized into EGFR-positive cells with greater efficacy than either naked Ad or Ad complexed with PP. Systemically administered PPE-complexed oncolytic Ad elicited significantly reduced immunogenicity, nonspecific liver sequestration, and hepatotoxicity than naked Ad. Furthermore, PPE-complexed oncolytic Ad demonstrated prolonged blood retention time, enhanced intratumoral accumulation of Ad, and potent therapeutic efficacy in EGFR-positive orthotopic lung tumors in comparison with naked Ad. We conclude that ErbB-conjugated and PEGylated PAMAM dendrimer can efficiently mask Ad's capsid and retarget oncolytic Ad to be efficiently internalized into EGFR-positive tumor while attenuating toxicity induced by systemic administration of naked oncolytic Ad.

706. Overcoming Manufacturing Challenges for Rapid Development of Genetic Nanomedicines from Discovery to Scale-Up

Mariam S. Assadian

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Nucleic acid therapies permit access to previously undruggable pathways and allow manipulation of cellular machinery to produce target proteins including antigens for vaccines. To overcome challenges of gene delivery, scientists are optimizing nanoparticles for delivery by fine-tuning their size, composition and surface properties. Although these efforts have yielded substantial results in the laboratory to date, a significant need exists for robust manufacturing technologies to transit these discoveries from lab to clinic. In this context, we present a microfluidic based NanoAssemblr™ platform for production of mL to L of nanoparticles that retains consistent quality, efficacy and safety profiles, at scale, throughout the development process.

Factor VII siRNA lipid nanoparticles (LNPs) were prepared on the NanoAssemblr™ Benchtop (Precision NanoSystems, Inc., Vancouver, Canada) and formulation parameters such as concentrations, flow rate ratio and total flow rate were optimized. These optimized process parameters were transferred onto the NanoAssemblr™ Blaze™ and 8X Scale-up System to scale this formulation to 100 mL and 1000 mL respectively. Physico-chemical properties and in vivo activity were measured for particles produced by each instrument to test consistency across the platform. Particle composition was determined by HPLC. Particle size and polydispersity was analyzed using dynamic light scattering and RNA encapsulation efficiency was determined from standard RiboGreen based assay. Finally, in vivo activity was tested by administering LNPs in wild-type mice by i.v. injection and measuring serum Factor VII levels.

We obtained LNP particles of about 60 nm (PDI <0.1) with encapsulation efficiency >95% on the NanoAssemblr™ Benchtop. No differences were observed in physicochemical properties of these particles when scaled-up by 10x on Blaze (100 mL) or by 100x on 8X

Scale-up System (1000 mL). The particles exhibited consistent lipid composition and N/P ratio within the target specifications. In addition, the particles manufactured across the microfluidic platform showed a similar dose-dependent gene knockdown, achieving > 90% reduction in protein levels at a dose of 1 mg/kg.

These studies demonstrated that the NanoAssemblr™ platform provides seamless scale-up and can produce large-scale volumes of lipid nanoparticles with consistent results. The 8X scale-up system can prepare up to 25 L of product 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.

707. Invivofectamine™ Rx Reagent: Novel Lipid Nanoparticle (LNP) for Therapeutic *In Vivo* mRNA Delivery to the Liver, Lung and Spleen for Gene Editing Application

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Messenger RNA (mRNA) has attracted a lot of attention recently as a new drug class to deliver genetic information. This excitement has been fueled by the fact that mRNA-based cancer immunotherapies and infectious disease vaccines have already entered clinical development in a very short span of time. The focus has been on the use of mRNA in treating liver diseases, metabolic diseases, cancer, neurological diseases, mRNA vaccine development, CRISPR gene editing and gene therapy. However, efficient delivery of mRNA is the major bottleneck that needs to be addressed and overcome in order for this field to explode and meet its exciting potential. We have developed novel Lipid nanoparticles (LNPs) through a Design of Experiment (DOE) approach to maximize the *in vivo* delivery efficiency of the mRNA. Our approach involves systematically designing an optimal DOE that takes into account both main and interaction effects, analyzing the experimental data using appropriate statistical techniques and generating response predictions from all potential combinations of the parameters. This iterative process is repeated till the optimal formulation is discovered. Adopting this approach provides us the flexibility to optimize formulations for organ specific delivery - Liver, Spleen, Lungs and Muscle (vaccines). 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 made significant progress moving through several generations of formulations where our current generation liver delivery LNP is 15,000X more potent than its first generation predecessor. Lung specific expression levels could be modulated by varying the dose of mRNA, and significant protein expression was sustained over the course of 48 hours following a single administration. Further optimization of biodistribution to achieve exclusive targeting to the lung is currently underway, and preliminary data indicates that variance in charge ratio enhances delivery and expression exclusively to the lung, and depletes expression in other organs. Finally, flow cytometry analysis was used to analyze mRNA delivery to specific cell populations within the lung and spleen. These novel lipid nanoparticles are well tolerated *in vivo*, with no qualitative gross toxicity, and quantitatively analyzed by a comprehensive cytokine profiling performed on murine serum samples.

Vector and Cell Engineering and Manufacturing

708. Validation of Scalable and Compliant AAV Production

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The evaluation of Adeno-associated virus (AAV) vectors in pre-clinical studies and potential applications in the clinic require scalable, high-yielding and compliant production. Even though much progress has been made in recent years, AAV production remains a challenge for translating basic research into clinic.

Here we compare the GMP-compliant production of recombinant AAV (rAAV) by transient transfection of HEK 293 suspension cells grown in serum-free medium with the rAAV production using adherent HEK 293 cells. Transfections of suspension cells were carried out via polyethyleneimine (PEI) based transfection. For the transfection of adherent cells, we applied calcium phosphate precipitation. The same 2-plasmid system was used for all productions. Additionally to the upstream process we did compare the purification of vectors using either iodixanol gradient centrifugation or immunoaffinity chromatography.

For this study rAAV2/9 expressing a GFP reporter gene were produced with suspension cells at a scale of one liter in orbital shaken bioreactors. A corresponding cell number of adherent cells were transfected and cultivated in cell factories. The viral genome titer of purified vector preparations was assessed by qPCR. The infectivity was determined by gene transfer assays and flow cytometry. To evaluate identity and purity of rAAV samples, we performed SDS-PAGE, or western blot analyses. Electron microscopy was applied to assess the ratio of full particles versus empty particles. As a final validation we are currently evaluating transduction efficiency and transgene expression in the striatum of adult mice. The preliminary results demonstrated that the production of rAAV particles using suspension cells and IAC yields viral genome titers of up to 10^{13} VG/L. These titers were equal or higher than titers achieved by production with adherent cells. Furthermore the in vitro infectivity of vectors produced with the novel process was significantly increased. By using an IAC capture step we could achieve highly pure vector preparations and a recovery of ≥70%.

The presented process allows the production of rAAV for pre-clinical and clinical trials, scalable, highly pure and compliant to GMP.

709. Ultra-Fast Purification of Viral Vectors

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Production of viral vectors at industrially-relevant scales is a costly and wasteful process which inflates the cost of exciting cell and gene therapies. Purification of these large protein complexes presents a particular challenge, with losses in excess of 70% reported. New methods and technologies are needed to address issues relating to yield, contaminant clearance, and product quality whilst improving overall process productivity.

At industrial scales, viral vector purifications are dominated by porous bead technologies, which require slow flow rates to allow proteins time to diffuse in and out of the pores. The result is large columns that are slow to run, expensive to replace and often provide unsatisfactory separation performance. Monolith structures or porous membranes are an alternative, but these suffer from low binding capacities and chemistries that are not optimised for vector applications, impacting both yield and resolution.

To address these limitations a nanofibre based technology platform is being developed for viral vector purifications. The advantage of the nanofibre structure is that it exhibits pore sizes in the range of 0.2-2μm compared to 15-40nm for traditional porous beads. This makes the material ideally suited for the capture of vectors such as Adeno Associated Virus (~20nm), Adenovirus (~80nm) and Lentivirus (~100nm). The effective surface area for capture is also ~5x greater than membrane absorbers used in a number of current processes ($10m^2/g$ compared to $2m^2/g$).

Here data will be presented demonstrating how the nanofibre technology can be used to improve the purification process for a range of viral vectors. Including how separation performance can be tailored to unique serotypes of Adeno-associated virus, Adenovirus, and Lentivirus viral vectors. Critically, the open nanofibre structure enables efficient viral capture at very high flowrates. This increases the amount of vector that can be purified per unit time, improving purification economics, and reducing the amount of time vector is exposed to harsh salt or pH environments. The result is a purification train that offers astonishing productivity improvements and superior separation.

710. A Simple and Scalable Method to Concentrate Measles Virus Glycoprotein-Pseudotyped Lentiviral Vectors Using Affinity Chromatography

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The ability to pseudotype lentiviral vectors with envelope glycoproteins from other viruses can alter their tropism which can allow for cell or tissue-specific targeting. For example, lentiviral vectors pseudotyped with measles virus hemagglutinin (H) and fusion (F) proteins allow transduction of quiescent lymphocytes, and the H glycoprotein can be engineered for retargeting. One problem with measles virus glycoprotein pseudotyped lentiviral vectors is low titer during production. This results in the need to make large volumes of the vector and to concentrate to appropriate titers. We previously reported a simple and scalable method to rapidly concentrate measles virus glycoprotein-pseudotyped lentiviral vectors using anion-exchange membrane chromatography. One remaining problem with this technique is the inability to selectively deplete contaminating cellular and media proteins. In this study, we examined the use of affinity chromatography using a nickel-NTA resin to concentrate lentiviral vectors pseudotyped with a polyhistidine (6xHis)-tagged receptor-blind measles virus H glycoprotein. We were able to recover over 30% of the input virus with an overall reduction in protein by over 1600-fold. This provides is a simple and scalable method for concentrating and purifying lentiviral vectors bearing envelope glycoproteins containing a 6xHis tag.

711. Translating Autologous Cell Therapy Clinical Development to Broader Patient Access Through Automation and Continuous Improvement

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Engineered autologous cell therapies are rapidly progressing through clinical development into commercialization with the potential of being successful therapeutic modalities for hematological malignancies. Nonetheless, the manufacturing processes and logistics associated with cell therapies are very intricate and unique when compared to the traditional biological product pipelines within the pharmaceutical industry. As such, it is essential that a clear strategic vision is in place to ensure that these cell therapies are scalable to deliver on their potential therapeutic benefits. One area of focus to ensure the sustainability of these therapeutics is continuous process improvement and automation. The manufacturing processes that are utilized in the production of autologous cell therapies are complex, open, labor intensive, and time consuming. When taken together, these factors limit the potential reach and scale of cell therapies due to the cost and time required to manufacture and deliver them to patients. Presented here is the optimization of a manufacturing process for anti-CD19 chimeric antigen receptor (CAR) T cells; specifically, the development journey of fully automated and closed processes to alleviate the scalability constraints and technical complexity of CAR T manufacturing (i.e. T cell enrichment/selection, expansion, and harvest). The role of automation and operational excellence in translating autologous cell therapy clinical development to broader patient access will be explored through their application to the development of an optimized CAR T harvest procedure.

712. The Cost Effectiveness of Manufacturing Antigen-Specific T Cells in an Academic GMP Facility

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Background: With the rapid surge in clinical trials for T cell immunotherapy, interest in GMP facilities at academic institutions

has also increased. Beyond the expensive fixed-costs associated with these facilities, the actual cost of manufacturing products is not well published, nor is the cost-effectiveness of doing so. **Methods:** We analyzed the general cost of manufacturing each antigen specific T cell product, number of products produced in the past 30 months, and how many of those T cell products were infused. All T cell products were captured in the analysis, including allogeneic virus-specific T cells, virus-specific T cells derived from cord blood, T cells specific for the tumor associated EBV antigens LMP1 & LMP2 (LMP-T cells), tumor-associated antigen-specific T cells targeting PRAME, WT1 and survivin, and autologous HIV-specific T cells. With the exception of the LMP- T cells, all products were manufactured using antigen presenting cells pulsed with overlapping peptide libraries. Manufacturing was performed by trained staff in the GMP facility. All products were manufactured under investigational new drug applications held with the Food and Drug Administration. **Results:** 123 products were received by the facility from 2013-2016. From these, 69% (85) were used in T cell product manufacturing and frozen for clinical use. The remaining 31% were either ineligible for the study, never made it to cryopreservation, or have yet to be manufactured. Of the 85 products available 54 T cell infusions were performed to 45 (53%) of the referred patients. 63% of blood products received went unused. We have calculated that the mean cost of an antigen-specific T cell product is approximately $10,371. Based on the number of T cell products that were manufactured for intended recipients but never used (n=40), $165,936 is spent per year manufacturing T cell products that will never be infused. **Conclusion:** A large percentage of patients enrolled on our clinical trials never receive T cells manufactured for them, at a high cost to us. To offset the high cost of manufactured-but-unused-T cells, we are investigating adding eligible, manufactured T cell products to our existing third party T cell bank. While many of these products can still be used for third party banks, determining donor eligibility prior to the blood draw is critical because products derived from ineligible donors cannot be utilized in the third party setting.

713. Rapid Characterization of Transient Transfection Conditions for Scalable Production of Adeno-Associated Virus (AAV) Using NanoSight NS300

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Transient transfection using a mixture of the cationic polymer polyethylenimine (PEI) and plasmid DNA is a common method for producing recombinant adeno-associated viral (rAAV) vectors. Transfection efficiency can greatly impact production of viral particles and is dependent upon the formation of DNA/polymer complexes. Several factors can affect complex formation including the choice of transfection media and PEI molecule, the concentration of DNA, the PEI/DNA ratio, the mixing conditions, and the incubation duration. Additionally, the formation of PEI/DNA complexes is scale dependent, as mixing characteristics can change across scales. A fast and reliable assay to assess the quality of transfection complexes would provide a checkpoint for efficient transient transfection process development and scale-up. Nanoparticle tracking analysis (NTA) is a quick and simple method to measure particle size distribution in aqueous solutions and could be used to monitor PEI/DNA complex formation. NTA uses light scattering to capture videos of nano-scale objects, moving under Brownian motion, and then calculates particle size distribution via the Stokes-Einstein equation. In this study, we evaluated the effect of PEI/DNA complex formation on rAAV production, via triple plasmid transfection of HEK293 suspension cultures. A constitutive GFP-rAAV transgene plasmid was created to measure the transfection efficiency and viral production by fluorescent microscopy and qPCR, respectively. Complex size distribution and concentration were measured by NTA using the NanoSight NS300 during the mixing and incubation period of complex formation. The effect of PEI/DNA complex formation on rAAV production was determined for several different PEI molecules, transfection media conditions, mixing strategy, scales, and incubation times. The correlations between complex size distribution, transfection efficiency, and rAAV cell culture yield were evaluated. We demonstrated that the NanoSight NS300 is a rapid and robust method to monitor PEI/DNA complex formation and is beneficial for improving the efficiency for transient transfection process development and scale-up.

714. Manufacturing Scale AAV Vectors: Quantification Strategy for Clinical Trials

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Background: Classical potency assignment for adeno-associated virus subtype 8 (AAV) clinical trial material aims to quantify the AAV vector by measuring the vector genome DNA. This approach often involves high assay variability, i.e. a coefficient of variability (CV) of max. 60%, and thus renders it difficult to control dosing in dose-escalation clinical trials. Aim: To develop a strategy for potency assignment based on surrogate measurement (AAV8 ELISA) supported by a complementary assay portfolio and consistent large scale manufacturing process. Methods: The production process for gene therapy vector (AAV8) in a HEK293 cell line was internally developed and scaled up to manufacturing scale for clinical trials. The AAV vector lots produced in pilot (200L) and manufacturing (500L) scale were fully characterized by qPCR, AAV8 ELISA, cryoEM, native agarose gel electrophoresis, and in vivo potency testing of the FVIII gene therapy vector in FVIII ko mice. Results: Methods qualification and/or validation showed a CV of max.10% for AAV8 ELISA, max.43% for qPCR, and max.1% for cryoEM. The lots produced in pilot and manufacturing scale achieved comparable and constant amounts of full AAV8 vector (73 ±3%) as measured by cryoEM. Since the AAV8 ELISA showed lower variability than qPCR, and the fact that production achieved a constant amount of full AAV8 in all preparations, this assay was used for potency assignment for these lots. The correctness of AAV8 ELISA potency assignment was confirmed in the biological activity assay by dosing

AAV8 vector based on the AAV8 ELISA. Conclusion: AAV8 ELISA appears suitable for potency assignment of clinical trial material to allow greater precision and consistency in dosing during trials, if manufacturing can demonstrate lot-to-lot consistency.

715. Downstream AAV Recovery Dramatically Increased by Non-Ionic Surfactant PF68

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In recent years, vectors based on the adeno-associated virus (AAV) have found increasing applications in experimental, preclinical, and therapeutic settings. Many of these rely on AAV preparations of high purity, yield, and concentration. The downstream process of AAV production primarily effectuates purification and concentration, however influences yield negatively by the loss of vector at each of the steps. It is therefore key to minimize the retention throughout this process and to maximize recovery. Increased as a vector core servicing multiple AAV technologies, we have in place a universal protocol that is independent of serotype, and provides consistent yields and purity. Briefly, following HEK293 triple transfection with producer plasmids and harvest of total lysate (media and cells), the downstream protocol consists out of enzymatic digestion, low speed centrifugation, tangential flow Filtration, iodixanol gradient centrifugation, and buffer exchange. The recovery rate in this process was established for serotypes AAV2/9, AAV2/5 and AAV2/8 to be between 16% and 18%, for AAV2/2, AAV2/4 and Anc80 at about 5%. The loss of the viral particles is most likely due to capsid dependent binding and adherence to the filter membrane and plastic.

Here, we evaluated Pluronic F68 (PF68) which is a non-ionic surfactant, used previously in formulations of AAV to prevent vector loss in injection devices by Bennett et al. in 2008. A total of 80 AAV preparations were made of different serotypes of AAV with a variety of transgenes by addition of PF68 at all the purification steps following TFF in order to keep PF68 concentration constant throughout the process, and compared to historical recovery data per serotype. Furthermore, we evaluated the effect of PF68 on qPCR titration and in vitro infectivity at various PF68 concentrations, and performed in process recovery assessment.

Results show that addition of in process PF68, recovery rates dramatically increased for all serotypes. AAV2/8 and AAV2/5 is around 54%, a three-fold increase, 34% for AAV2/9, 28% for AAV2/2, 37% for Anc80, a seven-fold increase, and 50% for AAV2/4. In *vitro* infectivity was not influenced, whereas qPCR was affected at high concentration of pluronic, however negligible at concentrations used in the production process.

716. Viral Safety for Cell and Gene Therapy Products

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Manufacturers of biopharmaceuticals have a responsibility to demonstrate that their products are safe from contamination by adventitious viruses. The approach to ensuring the viral safety of cellular and gene therapy products, however, is different than that used for monoclonal antibody and other recombinant protein products. Like other biological products, viral safety for cellular and gene therapy products requires careful screening of raw materials to ensure the absence of adventitious viruses. While many recombinant products can be manufactured without animal-derived raw materials, some cell therapies may require serum or other animal-derived materials for growth, increasing the potential for contamination by an animal virus. For traditional biological products, the manufacturing process includes robust viral clearance steps that have the potential to inactivate or remove viral contaminants, ensuring that a safety net exists in case a viral contaminant were to escape screening and enter the manufacturing process. The manufacturing process for a cellular product or a gene therapy vector may not provide opportunities for viral clearance, and any contaminant entering the process would not have an opportunity to be cleared.

Sensitive screening methods should be used to evaluate raw materials, including any virus or cell banks, for adventitious viruses. Molecular methods, including next generation sequencing, can determine the presence of viral nucleic acid and infectivity-based methods can be used to verify the absence of infectious viruses. The design of the testing scheme is based on risk factors for various types of viral contaminants; for example a bovine-derived raw material would be screened for bovine viral contaminants.

Data will be shown that demonstrate that in some cases, viral clearance can be achieved by steps of the manufacturing process, especially for non-enveloped viral vectors such as adeno-associated virus or adenovirus. Enveloped viral contaminants can be inactivated by process steps that contain detergent or that are at a low pH. In addition, large pore virus reduction filters can be used to retain large viruses that might be a potential contaminant, while a small viral vector passes through the filter.

Another approach to viral safety for cellular and viral gene therapy products is the use of methods such as UVC, filtration or high temperature short time (HTST) to inactivate or remove potential viral contaminants in cell culture media and other raw materials, thus providing an upstream barrier to contamination of the manufacturing process. Data will be provided to demonstrate the efficacy of these methods.

While the nature of cell and gene therapy products present a unique challenge for viral safety, careful selection and testing of raw material, inclusion of viral reduction steps where possible and the use of upstream barriers can result in products that are safe from adventitious viruses.

717. CD34+ Cells Enrichment Using a Closed Automated System for Ex Vivo Gene Therapy

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Ex vivo gene therapy for hematological disease is based on the purification of $CD34^+$ cells from mobilized peripheral blood (MPB) or bone marrow, the transduction of purified cells by retroviral vectors and the transplantation of the autologous gene-corrected cells to the patient with or without conditioning. In this study, we have evaluated $CD34^+$ cells enrichment from frozen apheresis with a fully automated cell processing instrument, the "CliniMACS Prodigy", compared to the conventional procedure used in current clinical trials for gene and cell therapy. We have determined the viability, the purity and the recovery of $CD34^+$ cells, using the double and single platform analysis. The quality of purified $CD34^+$ cells was also tested for transduction efficiency and engraftment capacity in NSG mice. The recovery of MPB_$CD34^+$ cells was similar for both selection procedures (Prodigy: 53±20%, n=8 *vs*. Washing cycles + CliniMACS: 52±29%, n=10) with identical $CD34^+$ cell purity (Prodigy: 84±6% *vs*. CliniMACS: 84±10%). MPB_$CD34^+$ cells purified by CliniMACS Prodigy were transduced with a GFP lentiviral vector and a dose-effect was obtained for VCN (2.9±1.4 after 1 hit of infection versus 4.7±0.8 after 2 hits of infection) or for frequency of GFP^+ cells (45±11% after 1 hit and 60±9% after 2 hits) in expanded $CD34^+$ cells. The human cell engraftment in NSG murine bone marrow at 12 weeks was around 37±20% with persistence of GFP expression in 53±25% of the engrafted human cells. With this new closed automated system, we therefore obtain the purification of $CD34^+$ cells with yield and quality comparable to the conventional procedure used in current clinical trial.

718. Production of Therapeutically Relevant Lentiviral Vectors for Clinical Studies

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The successful genetic engineering of patient-specific T cells with lentiviral vectors (LVV) expressing chimeric antigen receptors for late phase clinical trials and beyond requires the large- scale manufacture of high-titer vector stocks. However, their mass production remains a challenge and limits their potential therapeutic use and requires robust, scalable and regulatory compliant processes for industrial vector production. The state-of-the-art production of lentiviral vectors is based on 10- to 40-layer cell factories transiently transfected in the presence of serum. This manufacturing process is extremely limited by its labor intensity, open-system handling operations and its requirements for significant incubator space plus costs and patience risk due to presence of serum. To circumvent these limitations, this study aims to develop a stable and serum-free process to produce lentiviral vectors with PEI- mediated transfection. In addition, this study also focuses on the development of a production system not only using a GFP marker but also a therapeutically relevant transgene (CD20-CAR).

Therefore, three different cell lines (HEK 293, HEK 293T, HEK 293FT) were investigated concerning their productivity of LVV and their growing behavior in the in-house serum-free medium TransMACS. As part of this, Design of Experiment was used to investigate the optimal conditions for PEI/DNA-transfection. Furthermore, this statistical approach was used focusing an ideal ratio between the four construction 3rd generation plasmids (transfer plasmid CD20-CAR or GFP, envelope plasmid, packaging plasmids). In addition, different transfection enhancers were investigated concerning their enhancing effects on productivity comparing HEK cultures producing LVV encoding for GFP-marker or CD20-CAR.

The outcome of these experiments enabled the development of a robust HEK 293T based process to produce clinical relevant LVV under serum-free conditions. Furthermore, it provides an insight how therapeutic genes and the expression of its transgene can influence cell productivity.

719. Abstract Withdrawn

720. Stable Producer Cell Lines for Lentiviral Vector Manufacture

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Lentiviral vectors have been successfully applied in gene therapy clinical trials for an increasing number of conditions. Currently, the manufacturing scale of lentiviral vectors is a limiting factor in gene therapy and producing vector quantities sufficient to patient populations of more than a few hundred patients remains a significant technical challenge.

The majority of manufacturing processes involve the use of transient transfection of plasmid DNA into adherent human embryonic kidney 293T producer cells, grown in cell factories. This approach is of limited use in large scale vector manufacture due to the manual nature of the process, high costs and long lead times involved in obtaining plasmid DNA of sufficient quality. Adherent cell factories have poor scalability beyond low 10s of litres.

To meet the need for large scale vector manufacture GSK are establishing a cell line development process to generate stable lentiviral vector producer cell lines. Elimination of the transient transfection step during vector production greatly simplifies the process and reduces costs, whilst improving scalability and potentially the robustness of larger scale lentiviral manufacture necessary for the treatment of large patient populations.

721. Nanoceria-Loaded Injectable Hydrogels for Potential Age-Related Macular Degeneration Treatment

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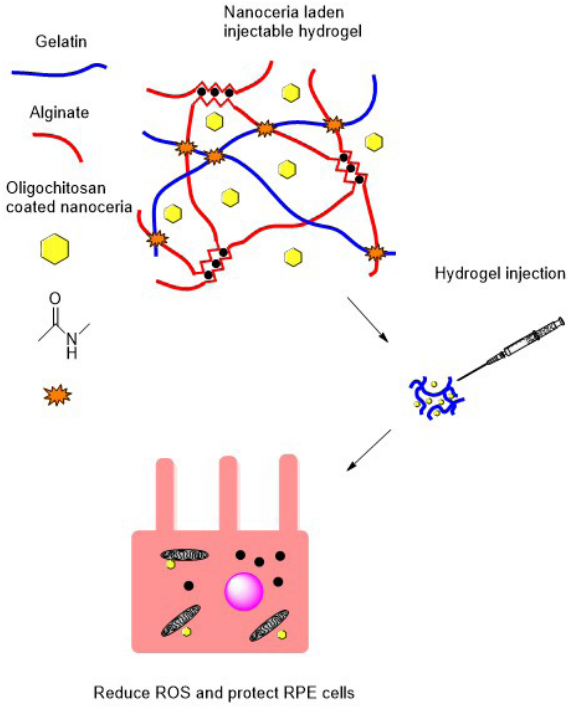
Alginate and gelatin were chemically crosslinked and ionic crosslinking of alginate moiety rendered a formation of injectable hydrogels. The water soluble oligochitosan coated cerium oxide nanoparticles

were loaded within injectable hydrogels as the antioxidative agent. The hydrogels behaved with moderate swelling and controllable degradation, and laden nanoceria was released by the function of degradation *in vitro* during two months of testing. The nanoceria loaded hydrogels exhibited robust antioxidation properties in oxygen radical absorbance capacity tests and reduced apoptosis of cells. The nanoceria laden injectable hydrogels are biocompatible and suppressed the induced inflammation response in ARPE-19 cells and inhibited expression of vascular endothelium growth factor in human umbilical endothelium, NIH 3T3, and B16F10 melanoma cell lines. This type of material has great potential for the treatment of oxidative stress related age-related macular degeneration.



Clinical Trials Spotlight Symposium

722. Gene Therapy for Beta Thalassemia: Initial Results from TIGET BTHAL Clinical Trial

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Gene therapy for beta-thalassemia, as an alternative cure to allogeneic HSCT, is based on the autologous transplantation of hematopoietic stem cells (HSCs) engineered by lentiviral vectors expressing a transcriptionally regulated human beta-globin gene. Our contribution to this field was devoted to the clinical development of a gene therapy protocol based on high-titer vector GLOBE, use of G-CSF and plerixafor as source of HSCs and a conditioning regimen based on treosulfan and thiotepa favoring efficient engraftment of corrected cells with reduced toxicity. On the basis of extensive efficacy and safety preclinical studies the clinical trial TIGET BTHAL (NCT02453477) was approved and started in 2015. The clinical protocol will treat 10 patients: 3 adults followed by 7 minors, with a staggered enrolment strategy based on evaluation of safety and preliminary efficacy in adult patients by an independent data safety monitoring board before inclusion of pediatric subjects. The chosen route of administration of gene modified cells is intraosseous in the posterior-superior iliac crests, bilaterally, with the aim of enhancing engraftment and minimizing first-pass intravenous filter. As of January 2017, seven patients with different genotypes (β^0/β^0, β^+/β^+ and β^0/β^+) have been treated with GLOBE-transduced $CD34^+$ cells at a dose of $>16x10^6$ cells/kg (max. $19.5x10^6$ cells/kg) and a VCN/cell ranging from 0.7 to 1.5. The procedure was well tolerated by all patients, with no product-related adverse events. Multilineage engraftment of gene-marked cells was observed in all tested peripheral blood and bone marrow samples. Polyclonal vector integrations profiles have been detected in the first tested 3 patients, with thousands of unique integration sites and no evidence of clonal dominance. The vector integrated with the expected genomic distribution, with the same top-targeted genes detected in other trials with LVs. So far, the clinical outcome indicates reduction in transfusion requirement in adult patients and greater clinical benefit in younger patients. Follow up analysis are ongoing and updated clinical outcome will be presented.

723. *SPK-9001*: Adeno-Associated Virus Mediated Gene Transfer for Hemophilia B at the Low Vector Dose Achieved Sustained, Continuous Factor IX Activity Levels Adequate for Endogenous Prophylaxis Preventing Bleeding Episodes and Reducing the Risk of Immune Response

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Introduction: Despite the ability of intravenous (IV) injections of clotting factors to restore hemostatic effect, low adherence to prophylactic regimens remains a major challenge (Schrijvers *et al.* 2016) in the hemophilia community. In addition, sustained levels of factor activity >12% may adequately eliminate joint bleeding (den Uijl IE *et al.* 2011). We are studying the ability of a single IV infusion of an investigational gene transfer product at a low dose level to obtain sustaining factor IX (FIX) activity levels adequate to prevent spontaneous hemarthroses while reducing or controlling capsid-directed T-cell responses. **Methods**: We infused an investigational product, *SPK-9001*, with a bioengineered recombinant AAV hepatotropic capsid (AAV-Spark100) and a codon-optimized, single-stranded transgene encoding FIX-Padua that confers enhanced specific activity compared to wild-type FIX (Simioni *et al.* 2009) in hemophilia B males. Data on the number of bleeding episodes and FIX injections in the year prior to enrollment were compiled. Laboratory values, AAV immune monitoring, frequency of bleeding episodes, and FIX consumption were evaluated after vector infusion. **Results**: As of 1/25/2017 (cumulative total follow-up >306 weeks), 9 males ages 18-52 years with baseline FIX:C ≤2% of normal and Spark100 NAb titers ≤ 1:1 have reached over 12 weeks of follow-up after the infusion of *SPK-9001* at a dose of $5x10^{11}$ vg/kg. The mean steady-state FIX:C of ~29% allowed discontinuation of prophylaxis occurring the day after vector infusion in all cases. *SPK-9001*-induced FIX activity levels in each participant plateaus about 12 weeks post infusion and is sustained and stable thereafter. One participant, with significant underlying hemophilic arthropathy, reported two separate occasions for suspected hemarthrosis. Otherwise, no participants have experienced any bleeding events amounting to a cumulative factors reduction of >1.4 million IUs. Two out of 9 infused participants observed an asymptomatic increase in hepatic transaminases resulting in a tapering course of prednisolone treatment, starting at 60 mg/day. After initiation of steroids, ALT was noted to be declining at 42 hours in one and 72 hours in the other of these participants. Other than noted above, there were no participants reported study-related SAEs or development of FIX inhibitor. **Conclusion**: We report the highest sustained plasma FIX activity levels consistently above 12% across all nine participants and the lowest (22%) incidence rate of initiation of steroid therapy triggered by increase in ALT and/or positive IFN-γ ELISPOT after a single IV infusion of *SPK-9001* at a dose of $5x10^{11}$ vg/kg. To date, these sustained levels of expression have permitted safe discontinuation of prophylaxis in all participants and prevention of bleeding. Furthermore, in both participants who were treated with steroids, FIX activity levels stabilized at levels adequate to prevent bleeding episodes over the current period of observation.

724. TET2 Gene Disruption via Lentiviral Integration Promotes CAR T Cell Expansion and Persistence

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Adoptive cell transfer therapies with chimeric antigen receptor (CAR) T cells that target tumor antigens can achieve remissions in patients with hematologic malignancies (PMID 26333935). A major obstacle to the broader clinical application of this technology has been the limited expansion of CAR T cells and their rapid disappearance following infusion, especially in the setting of immunosuppressive diseases such as chronic lymphocytic leukemia (CLL). Here we report the remarkable case of a seventy-eight-year-old patient with CLL who was treated with CAR T cells that target CD19 (CTL019). Upon administration of polyclonal autologous CTL019 cells, delayed CD19-specific immunity was observed in the peripheral blood, bone marrow and lymph nodes, accompanied by complete remission. Deep sequencing of the variable region of the T cell receptor-beta chain showed that at the height of this patient's anti-tumor response, approximately ninety-eight percent of CD8+ CTL019 cells originated from a single clone. Subsequent analysis of lentiviral integration sites revealed that proviral insertion of the CAR transgene disrupted one allele of the methylcytosine dioxygenase *TET2* in this clonal CD8+ CAR T cell population. Accordingly, evaluation of polyadenylated TET2 RNA populations demonstrated the appearance of new forms that spliced into the vector and terminated, truncating the encoded protein. *TET2* insertional mutagenesis led to decreased cytosine hydroxymethylation and acquisition of an epigenetic profile consistent with altered T cell differentiation. Retrospective analysis of the differentiation state of *ex vivo* CTL019 cells from the above patient indicated that they were of an early memory phenotype at the peak of efficacy, which was distinct from other patients who had long-term durable responses to this therapy that were characterized by effector memory CAR T cell differentiation. Knockdown of TET2 recapitulates the effect of insertional disruption on the differentiation state of both total and CAR+ CD8+ as well as CD4+ human T cells from several normal donors, implicating DNA demethylation as an epigenetic regulator of T lymphocyte fate. This discovery suggests that adoptively-transferred T cells with vector integration sites in a specific gene locus are strongly selected because these integrations may promote their anti-tumor efficacy. Thus, targeting the epigenome using small molecules, highly efficient site-directed transgene integration strategies or other genetic engineering approaches may improve the efficacy and persistence of cellular therapies for cancer.

725. ZUMA-1 Pivotal Phase 2 Trial of Axicabtagene Ciloleucel (axi-cel, KTE-C19; Anti-CD19 CAR T Cells) in Patients (pts) with Refractory Aggressive Non-Hodgkin Lymphoma (NHL)

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Background: Pts with refractory aggressive NHL have poor outcomes with available therapies (Crump, ASCO 2016). ZUMA-1, the 1st multicenter trial of anti-CD19 chimeric antigen receptor (CAR) T cells in refractory aggressive NHL showed 43% ongoing complete response (CR) at 12+ mos in phase 1 (Locke, Mol Ther 2016). We present results of a phase 2 prespecified interim analysis. **Methods:** Pts from two cohorts, DLBCL (C1) and PMBCL/TFL (C2), received 2 × 10^6 anti-CD19 CAR T cells/kg after a low-dose Cy/Flu conditioning regimen. Pts ≥18 years with ECOG PS 0-1, refractory disease (PD or SD as best response to last line of therapy, or PD ≤12 mos after autoSCT), and adequate prior therapy were included. This analysis assessed early efficacy in 51 C1 pts with ≥3 mos follow up. **Results:** Among 111 enrolled pts, 101 received axi-cel. As of Aug 24, 2016, 51 pts in C1 and 11 pts in C2 were eligible for analysis. Axi-cel was successfully manufactured in a centralized facility in 99% of enrolled pts with a turnaround time of 17 days. With an ORR of 76% in C1, the primary endpoint was met (P<0.0001 compared with historical control, 20%); 92% of responses occurred within 1 mo. Overall, 44% of pts had ongoing responses (CR, 39%) at 3 mos. Responses were seen across key covariates including refractory subgroup, stage, IPI and age. AEs are shown in table 1. Peak CAR T cell expansion associated with ongoing response at 3 mos (P=0.004) and grade ≥3 neurological events (NEs; P=0.02). Pts with grade ≥3 cytokine release syndrome and NEs had increased levels of IL-15 (P=0.04 and 0.006) and IL-6 (P=0.001 and 0.0003). **Conclusions:** In this trial of CAR T cell therapy in refractory aggressive NHL, axi-cel increased CR rate 6-fold over historical outcomes in SCHOLAR-1. Efficacy was associated with peak CAR T levels. Central manufacturing, logistics, and AE management were successful across sites. Axi-cel demonstrated significant clinical benefit in pts with no curative treatment options.

Table 1. Baseline Characteristics and Results From ZUMA-1 Phase 2

	Cohort 1 DLBCL (n=51)	Cohort 2 PMBCL/TFL (n=11)	Total (N=62)
Baseline characteristics			
Median age (range), years	58 (25-76)	57 (28-68)	58 (25-76)
Age ≥65 years, n (%)	12 (24)	2 (18)	14 (23)
Male, n (%)	37 (73)	9 (82)	46 (74)
ECOG performance status 1, n (%)	36 (71)	4 (36)	40 (65)
Received ≥3 lines of prior therapy	31 (61)	10 (91)	41 (66)
Refractory subgroup, n (%)			
Refractory to ≥2 lines of prior therapy	40 (78)	9 (82)	49 (79)
Relapsed within 12 mos of ASCT	10 (20)	2 (18)	12 (19)
Response			
Overall objective response, n (%)	39 (76)	10 (91)	49 (79)
(95% CI) P value[a]	(63, 87) P<0.0001	(59, 100)	(67, 88)
Best response, n (%)			
CR	24 (47)	8 (73)	32 (52)
PR	15 (29)	2 (18)	17 (27)
Safety[b]	**Cohort 1 (n=73)**	**Cohort 2 (n=20)**	**Total (N=93)**
Grade ≥3 adverse event,[c] n (%)	68 (93)	18 (90)	86 (92)
Grade ≥3 TEAEs of clinical interest, n (%)	-	-	-
Cytokine release syndrome[d]	10 (14)	2 (10)	12 (13)
Neurologic events[d]	8 (25)	9 (45)	27 (29)
Fatal events excluding PD 2 of 3 axi-cel-related[e]	1 (1)	2 (10)	3 (3)

[a]Exact binomial test comparing observed ORR in DLBCL to a historical control assumption of 20%. [b]Safety outcomes are shown for 93 treated subjects in C1 and C2 with ≥1 mo follow. [c]Occurring in ≥20% of patients. [d]Combined terms. [e]Two grade 5 axi-cel-related events were hemophagocytic lymphohistiocytosis and cardiac arrest in the setting of cytokine release syndrome. TFL, transformed follicular lymphoma; PMBCL, primary mediastinal B cell lymphoma; TEAE, treatment-emergent AEs.

726. A Potential Terminator of Multiple Myeloma: Myeloma: LCAR-B38M CAR-T Cells Achieved Unprecedented High Rate of Continuous Complete Remission (CCR) in Refractory or Relapsed Multiple Myeloma Patients

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Chimeric Antigen Receptor Engineered T cell therapy (CAR-T) is a novel immunotherapy for cancer and has been clinically validated in the treatment of acute lymphoblastic leukemia (ALL) and lymphoma by targeting CD19. However, the slow progress of CAR-T therapies targeting other molecules raised skepticism if the initial success of CAR-T can be extended to effective treatment of other liquid cancers or solid cancers. Here we report an encouraging breakthrough of treating multiple myeloma (MM) using our proprietary CAR-T modality targeting BCMA. Legend Biotech discovered bispecific LCAR-B38M CAR-T technology and conducted a single arm phase I/II clinical trial to assess the safety and efficacy of the novel technology. A total of 19 patients diagnosed with refractory/relapsed multiple myeloma had been treated. All these patients had experienced previous failure of three or more rounds of first-line therapy. PBMC of the patients were collected by apheresis and LCAR-B38M CAR-T cells were prepared by lentiviral gene transfer. The patients were pretreated with cyclophosphamide (300 mg/m2) for 3 days (days -5,-4,-3) prior to infusion of the LCAR-B38M cells. A split-dose cell infusion schedule were used (day 0, 2 and 6) to enhance the safety. The median number of infused cells was 4.7 (0.6 ~ 7.0) × 106/ kg. The median follow-up time is 183 (60 ~ 293) days. Among 19 patients who completed infusion, 7 patients were followed for longer than 6 months. 6 of the 7 patients achieved the most strict criteria of CR, the immunophenotypic CR as well as MRD negative status. The rest one patient temporarily reached PR but relapsed soon after receiving large dose of steroid around surgical treatment for an unexpected meningioma onset. The steroid might compromised the therapeutic outcomes of the CAR-T cells and the patient died 8 months later as the disease progresses. There are also 12 patients who are followed less than 6 months are still evaluated as near CR (CR but immunofixation positive) based on modified EBMT criteria. All these patients are showing a progressively decreasing trend of M-protein despite still positive on sensitive immunofixation, thus have substantial chance to eventually meet CR criteria with time. Currently all these 18 patients are free of myeloma-related biochemical and hematologic abnormalities in the most recent follow-up. The most common adverse events are acute (within 15 days post-treatment) cytokine release syndrome (CRS), which was appeared in 14 (74%) patients (5 cases (26%) free of evident CRS need any treatment, 9 case of grade 1, 2 cases of grade 2, 1 cases of grade 3 and 1 single case of grade 4). The 2 most serious CRS cases (grade 3 and grade 4) experienced capillary leakage syndrome manifested as hypovolemic shock, pulmonary edema and pleural effusion. All 2 cases recovered after treatments with Tocilizumab, vasopressors and diuretic agents. The main chronic (> 30 days) adverse reaction was hypogammaglobulinemia. In summary, an overwhelmingly positive response (100% ORR) to LCAR-B38M CAR-T cells was observed in refractory/relapsed myeloma patients. 18 out of 19 (95%) patients reached CR or near CR status without even a single event of relapse in a median follow-up of 6 months. Most patients experienced very mild adverse events/CRS and 26% of patients are even free of CRS. Thus, we confidently believe that the outstanding safety and efficacy profile of the innovative LCAR-B38M cells have established itself the best-in-class CAR-T product in treating multiple myeloma.

727. A Phase III Clinical Results of INVOSSA™ (TissueGene-C): A Clues for the Potential Disease Modifying OA Drug

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Invossa™ (TissueGene-C) is a cell and gene therapy for osteoarthritis that contains non-transformed and transduced chondrocytes by the ratio of 3:1. The transduced cell employs *ex-vivo* gene delivery via a retrovirally transduced chondrocytes that overexpress transforming growth factorβ1 (TGFβ1). The randomized double blind, multi-center, placebo-controlled phase III trials were conducted to determine both safety and efficacy in patients with knee osteoarthritis. Participants (n = 156) with a confirmed diagnosis of knee osteoarthritis by X-ray and MRI were randomized into the treatment group (Invossa™, n = 78) and the control group (saline, n = 78). The primary evaluation parameters were comparison of changes after 52 weeks from the baseline of International Knee Document Committee (IKDC) and VAS. The secondary parameters were evaluated by Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) and Knee Injury and Osteoarthritis Outcome Score (KOOS), Joint Space Width (JSW) with X-ray, Whole Organ Magnetic Resonance Imaging Score (WORMS) with MRI, and biomarkers from serum and urine samples. The observation period was one year after a single injection. The primary parameters, IKDC total score and in VAS score at 52 weeks were statistically significant (IKDC: $P<0.001$; VAS: $P<0.001$). The secondary parameters, WOMAC and KOOS, also showed statistically significant improvement in 1 year follow up after a single injection of Invossa™. With the quantitative MRI analysis, the Invossa™ treatment group showed improvement for the bone area and cartilage thickness.

In summary, Phase III study indicated that Invossa™ treatment improved pain, sports activities, and quality of daily life in patients with knee osteoarthritis when compared to the placebo control. The Invossa™ treatment also showed clues of potential disease modifying OA drug.

Rational Design and Analysis of AAV Vectors

728. Analysis of the AAVrh.10 Capsid Structure and Its Antigenic and Receptor Interactions

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In recent years the use of vectors based on Adeno-associated virus rhesus isolate 10 (AAVrh.10) has grown rapidly for a variety of different gene therapy trials. The advantages of AAVrh.10 include its high transduction efficiency of the CNS in vivo and the low percentage of pre-existing neutralizing antibodies in the human population. However, despite its increasing application in various gene therapy trials, little is known about the basic biology of AAVrh.10. Here we present the AAVrh.10 capsid structure determined by cryo-electron microscopy (cryo-EM) and three-dimensional image reconstruction (cryo-reconstruction) with a resolution of 3.9 Å. The capsid structure of AAVrh.10 is similar to AAV8 with which it shares an amino acid sequence identity of 94%. However, clear differences between both structures exist on the capsid surface at the previously described AAV VP variable region I (VR-I) and VR-IV which are located on the 2/5-fold wall and protrusion surrounding the 3-fold symmetry axis, respectively. These regions of the capsid are associated with tissue transduction and antigenicity for many AAV serotypes. However, many antibodies developed against AAV8, cross-react with AAVrh.10. Mutagenesis of those antigenic sites led to AAVrh.10 vectors that escape these antibodies while maintaining infectivity.

Towards identifying a glycan receptor for AAVrh.10, the virus was screened on a glycan microarray and observed to interact with a sulfated LacNAc, Gal(b1-4)GlcNAc-6-sulfate(b1-3)Gal(b1-4)GlcNAc-6-sulfate. The binding site for this glycan on AAVrh.10 capsids was determined by cryo-reconstruction to 4.3 Å. A comparison of AAVrh.10 structures with and without glycan revealed an additional density on the capsid surface at the 2-fold symmetry axis for the complex structure. This binding site differs from those described for other AAV-glycan interactions which have been mostly localized to the 3-fold axis and the protrusions which surround it. Mutational analysis of residues in that glycan binding pocket confirm its importance for glycan interaction, cell binding, and transduction.

A comparative analysis of AAVrh.10 with other AAV capsid structures, its engineering to escape antibody recognition, and the properties of the AAVrh.10 2-fold axis which provide the unique features required for LacNAc binding will be presented.

729. Using Novel Engineered AAV Capsids with High Efficiency Retrograde Transport to Map and Modulate the Function of Specific Neuronal Subpopulations Projecting to the Lateral Striatum

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Viral targeting of specific neurons projecting only to specified areas within the CNS has long been hampered by a lack of suitable transgenic animals. An alternate approach is to infect the neurons of interest retrogradely from the target using viral vectors. Inefficient retrograde transport of viral capsids and neuronal toxicity when using pseudo-typed rabies virus has however limited this approach. In order to create a biological tool to remedy these shortcomings we have created novel AAV capsids using rational systemic screening of a peptide library, derived from proteins known for synaptic targeting and retrograde transport. In this screening process we identified, among others, one serotype (MNM-004) with excellent retrograde transport capacity. When injected into the lateral striatum, MNM-004 displayed highly efficient retrograde transport to several known nuclei projecting to the targeted region. These included the frontal cortex, intralaminar thalamic nuclei, parafasicular thalamic nuclei, zona inserta, and amygdala, many of which play a poorly understood role in movement disorders like Parkinson's disease. Importantly, unlike rabies mediated retrograde transport the MNM-004 viral vector does not appear to be toxic in any of the labelled neuronal populations, allowing for long term studies of these specific subpopulations. As a proof of concept we injected cre-recombinase expressing MNM-004 into the striatum of Sprague-Dawley rats while a vector expressing an inducible mCherry-Flex-GFP construct was injected into the intralaminar nuclei of the thalamus, which may play an important but as of yet undefined role in Parkinons disease. As expected only neurons within the intralaminar thalamic nuclei stained positive for GFP while surrounding thalamic nuclei only stained positive for mCherry confirming specific targeting of GFP to only the intralaminar thalamic nuclei. Combining this approach with chemogenetic designer receptor DREADDs allows for nuclei specific or even cell specific modulation of neuronal subpopulations projecting only to a specific brain region. We used this approach to elucidate the function of the basolateral amygdala by injecting a cre expressing MNM-004 into the striatum of Sprague-Dawley rats, followed by an injection of AAV-8 viral vectors containing DREADDs into the amygdala. As expected only neurons within the basolateral amygdala expressed the DREADD receptors as these neurons are known to project into the striatum. Modulating these neurons through systemic injections with DREADD ligand CNO induced a significant fear and anxiety phenotype for several hours in the injected rats. This novel approach allows for non-toxic modulation and tracing of specific neuronal sub populations based on their projection patterns to different regions within the CNS, making it an invaluable tool for elucidating complex projection networks in a variety of disorders.

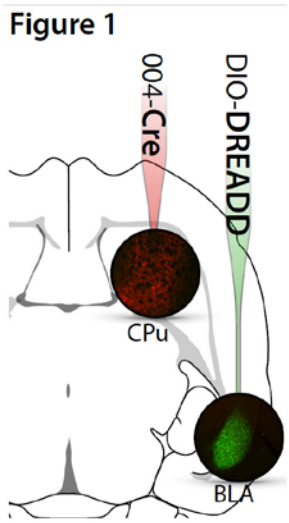

Figure 1. Cre-recombinase expressing MNM-004 AAV (red) injected into the striatum (Cpu) with inducible Flex-DREADDs (green) injected into the Blasolateral Amygdala (BLA). MNM-004 serotype displayed excellent retrograde transport into the BLA.

730. HBoV1 Capsids Facilitate rAAV Genome Transduction in Newborn Ferret Airways

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Human bocavirus type-1 (HBoV1) is highly tropic for the apical membrane of human airway epithelial and chimeric recombinant vectors, rAAV2/HBoV1, composed of an adeno-associated virus (rAAV2) genome and HBoV1 capsid can efficiently transduce human airway epithelia, making them attractive gene therapy vectors for lung diseases such as cystic fibrosis. However, preclinical development of rAAV2/HBoV1 vectors has been hindered by the fact that humans are the only known host for HBoV1 infections. Here, we report the lung of newborn and juvenile ferrets is highly transduced with rAAV2/HBoV1. rAAV2/HBoV1 efficiently transduced the airways of newborns (day-3 to day-7 old) and day-29 old ferrets with transgene expression predominantly in the distal airways. Interestingly, in vivo, ex vivo, and in vitro models of ferret proximal airway demonstrate that rAAV2/HBoV1 poorly infects this region of the ferret airway. While transgene expression declined proportionally with growth of animals following infection at 7 days of age, reinfection of kits with rAAV2/HBoV1 at 29 days showed no decrement in transduction as compared to naive animals. Studies on vector binding and endocytosis with polarized airway epithelial culture revealed that the lack of effective vector endocytosis is the main cause of the inefficient transduction in vitro, consistent to our previous finding that the HBoV1 and rAAV2/HBoV1 vector preferentially infect fully differentiated human airway epithelium. These studies lay the foundation for clinical development of rHBoV1-based vectors for lung gene therapy in cystic fibrosis.

731. Facile Capsid Pseudotyping via Unnatural Amino Acid (UAA) Based Site-Specific Modification of AAV2 and AAV-DJ Capsids and Engineering of AAVs

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Recombinant Adeno Associated Virus (rAAV) vectors are amongst the most widely used vectors for gene therapy. Although favorable due to their lack of pathogenicity and ability to infect both dividing and non-dividing cells while persisting as extrachromosomal elements, engineering AAVs with novel surface properties has been challenging. For instance, AAV capsid proteins are typically inflexible to insertion of large peptides or biomolecules making it difficult to add novel effectors onto the capsid without heavily compromising on the viral titer or the infectivity. Ability to engineer capsids could also potentially enable approaches to address AAV immunogenicity, in particular the presence of AAV neutralizing antibodies present in the serum which is a major obstacle in therapeutic applications. Our study aims to provide potential solutions to these problems. In order to overcome the hurdle of capsid modifications, we have engineered an approach utilizing unnatural-amino acid mediated incorporation of bio-orthogonal chemical handles onto AAV capsid proteins that enables facile viral surface modifications. We began by replacing specific surface residues with the nonsense codon (TAG). Using an orthogonal UAA specific tRNA/aminoacyl-tRNA synthetase (tRNA/aaRS) pair that recognized the nonsense codon, the UAA, N-epsilon-((2-Azidoethoxy) carbonyl)-L-lysine was cotranslationally incorporated into the three capsid proteins VP1-3. The virus thus generated can be coupled with alkyne-linked novel effectors via click chemistry, enabling facile capsid pseudotyping. We demonstrated successful incorporation of the UAA into the viral capsid via the tethering of an alkyne-fluorophore followed by transduction of cells and also via SDS-PAGE. In addition, we demonstrated tethering of alkyne-tagged oligonucleotides onto the virus via selective capture on DNA array spots bearing complementary oligonucleotides. Finally, we integrated Cas9 payload delivery into the UAA-AAVs, and demonstrated no loss of activity and retention of full genome engineering functionality. In order to engineer resistance to serum based AAV neutralizing antibodies, we next screened a variety of alkyne-linked small molecules and polymers that were added onto the AAV via click chemistry. We were able to identify polymers that provided a high degree of resistance and thus engineered AAVs had nearly unchanged activity at progressively increasing serum concentrations whereas the wild type virus was completely neutralized. Moreover, we went on to utilize this modified virus to deliver a CRISPR-Cas payload and demonstrated several fold higher editing rates as compared to the wild type virus across various serum dilutions. We are currently in the process of demonstrating it *in vivo*. Taken together, we believe that our UAA-AAVs will open the door to widespread programmable pseudotyping of the AAV capsid while the shielding of

AAVs by lipids could potentially improve the efficiency of AAV based gene therapy and eliminate the need for immunosuppressive agents frequently administered during the process.

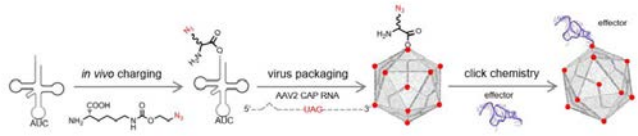

Figure: Schematic of approach for addition of a UAA to the virus capsid and subsequent click-chemistry based chemical linking of an effector to the UAA.

732. Bioinformatics Design of rAAV-Compatible MiniPromoters for Cell Type-Restricted Expression

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A main limitation of recombinant adeno-associated virus (rAAV) vectors in gene therapy is restricted payload capacity. With constrained vector size, most AAV-based gene therapies use small, ubiquitous promoters (e.g. CMV) to drive transcription of therapeutic genes. The use of ubiquitous promoters driving expression in off-target cells has the potential to produce undesired effects. In this context, designing compact promoter sequences that restrict expression of therapeutic genes to the most clinically relevant cells is an important research goal.

Previously, we have shown the capacity to design compact, selective promoters (MiniPromoters) targeting specific cells within the brain and eye. The early MiniPromoter design process involved three manual steps: 1) identification of an endogenous gene with restricted expression in the target cells; 2) annotation or prediction of *cis*-regulatory regions (RRs) of the gene (i.e. promoter(s) and enhancers); and 3) assembly of a subset of the RRs into a MiniPromoter sequence. We are developing *OnTarget*, software to automate key steps of the design process, with the goal of empowering the research community to design their own MiniPromoters.

Once an endogenous gene is identified, *OnTarget* provides users with a structured workflow resulting in MiniPromoter design. The system integrates publicly available genomics data that informs the delineation of RRs. This process begins with the identification of a promoter region. Promoter annotations are obtained from experimentally determined transcription starts sites across >200 human cells and tissues profiled in the FANTOM5 project. Additional RRs are sourced from a variety of relevant data sources, which can be selected by the user, such as ChIP-seq, DNase-seq, computational predictions, etc. The system thus allows the compilation of a variety of potential RRs supported by different forms of evidence.

In the next step, users bring together a subset of the RRs that fit within the available space (e.g. <2.7 kb). With the support of *OnTarget*, the time required for compilation is minimized, allowing users to focus on the selection and refinement steps. In the refinement stage, individual components of the design are finalized, including the selection of the boundaries of RRs, as well as the introduction of specific sequence alterations. For the latter, the system is designed to assist users in the modulation of transcription factor binding sites (strengthening or weakening predicted binding).

The *OnTarget* project confronts one of the major challenges of gene therapy: the restriction of gene expression to target cells. The presentation will include examples of MiniPromoter-driven expression, as well as a guide through the *OnTarget* workflow. We anticipate that *OnTarget* will reduce costs for designing compact RRs, increase capacity for selective delivery of gene therapies limiting off-target side effects for the patients, and advancing the analysis of human RRs.

733. Structural Mapping of AAV9 Antigenic Sites and the Engineering of Immune Escape Variants

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A recognized hindrance to Adeno-associated virus (AAV)-mediated gene therapy is the humoral immunity existing in patients, which typically ranges from 40% to 80% in epidemiological studies. These antibodies greatly reduce the efficacy of gene therapy transfer via neutralization, and limit the number of potential recipients. This study aimed to define the interactions between anti-AAV9 antibodies and AAV9, a serotype showing promise in the treatment of congenital CNS or skeletal muscle disorders, to assist in the development of recombinant vectors (rAAV9) able to escape neutralizing antibody recognition. Utilizing cryo-electron microscopy (cryoEM) and single particle image reconstruction, the antigenic epitopes of fragment antibodies (Fabs) for four mouse anti-AAV9 monoclonal antibodies (MAbs) have been determined to 11 to 16Å resolution. These antibodies, HL2368, HL2370, HL2374, and HL2372, which have the have the ability to neutralize AAV9 infection *in vitro* and *in vivo*, bound to the 3-fold axis region of the capsid, with the exception of HL2372, which bound to the region surrounding the 5-fold axis of symmetry. These two epitope regions overlap previously defined epitopes for other AAVs, confirming antigenic footprint commonalities between serotypes. Pseudo-atomic modeling identified specific interacting residues between Fabs and the capsid surface within these footprints, to inform the development of mutant AAV9 capsids capable of escaping recognition and neutralization by the parental MAbs, polyclonal serum from NHPs in a preclinical trial for Pompe's disease utilizing AAV9, and polyclonal human donor serum positive for wtAAV9 recognition. These rAAV9 variants will aid further development of AAV9-based gene delivery vectors able to escape pre-existing immunity against wild-type vectors, and increase the number of potential recipients of rAAV9-mediated gene therapy.

734. Efficient Protection from Pre-Existing Antibodies and Superior Correction of Hemophilia B with Exosome-AAV Vector Mediated Liver Gene Transfer

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Adeno-Associated Virus (AAV) vectors have emerged as the most efficient delivery system for gene transfer. In particular, recent data from clinical trials of gene transfer for hemophilia demonstrate the therapeutic potential of this gene transfer platform. However, vector-directed immune responses remain an important limitation to the widespread use of AAV in the clinic. To this end, high vector doses have been associated with a higher risk of triggering anti-capsid CTL responses. Additionally, anti-AAV pre-existing immunity can prevent liver transduction even at very low neutralizing titers. Here, we described the use of exosome-associated AAV vectors (exo-AAV) as robust liver gene delivery system that could help overcome AAV vector immunogenicity. We first evaluated the efficiency of standard AAV8 vectors expressing human factor IX (hF.IX) and exo-AAV8-hF.IX mediated liver gene transfer to evade pre-existing antibodies to the capsid. Animals were pre-immunized with 0.5 mg or 2 mg of intravenous immunoglobulin, resulting in an anti-AAV neutralizing antibody titer of ~1:3 and ~1:10, respectively. Exo-AAV8 gene delivery allowed to efficiently target the liver, protecting the vector from pre-existing antibodies and resulting in comparable level of hF.IX expression to naïve animals treated with standard AAV8 vectors. We next evaluated *in vivo* efficiency of liver targeting of standard AAV8 or standard AAV5 and exo-AAV8 or exo-AAV5 expressing hF.IX in naive animals. We observed a significant enhancement of transduction efficiency for both exo-AAV5 and exo-AAV8 vectors in both female and male mice. Interestingly, in Hemophilia B KO mice treated with $4x10^{10}$ vector genomes/kg of exo-AAV8 vectors, a dramatic increase (~1 log) in transgene expression (~97% of physiological levels of hF.IX, 4853.8 ng/ml, n=4) was observed compared to treated animals with standard AAV8 vectors (~7% of physiological levels of hF.IX, 373.5 ng/ml, n=3). Restoration of clotting activity was confirmed with an aPTT assay . In conclusion, exo-AAV vectors present superior liver transduction efficiency and resistance to anti-capsid antibodies compared with standard AAV preparations. Exo-AAV vectors therefore also represent a potential efficient platform to obtain efficient liver transduction at low vector doses, thus reducing the risk of induction of capsid-directed T cell responses.

735. Design of an AAV9-Based Gene Therapy Vector Able to Evade Neutralization by a Novel, Potent α-AAV9 Neutralizing Antibody

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While the field of gene therapy utilizing adeno-associated viral vectors has significantly advanced in recent years, both pre-existing and induced immune responses to the capsid continue to be a concern. Pre-existing neutralizing antibodies (NAbs) against the AAV capsid reduce the number of patients eligible for gene therapy, and NAbs generated following vector administration preclude patients from receiving a second dose. Through the isolation of anti-AAV antibodies and mapping of their neutralizing epitopes, we aim to rationally design novel AAV vectors that can evade these humoral responses. To isolate novel anti-AAV9 antibodies, we immunized Balb/c mice with AAV9 vector and generated hybridomas. From this panel of hybridomas, the anti-AAV9 antibody designated PAV9.1 was selected for further study. AAV9 binding was confirmed by ELISA and dot blot, and we determined that PAV9.1 recognizes a conformational epitope on the capsid surface by both methods. PAV9.1 was also screened for reactivity against additional AAV serotypes 1, 2, 5, 6, 8, rh10, and rh32.33 by ELISA and was found to be specific for AAV9. A NAb antibody assay in Huh7 cells determined that purified PAV9.1 is a highly neutralizing antibody with a titer of 1:81,920. To map the epitope of PAV9.1, we generated PAV9.1 Fab, which was then complexed with AAV9 vector, plunge frozen, and imaged to generate a low resolution reconstruction using traditional cryo-EM methods. The reconstruction identified PAV9.1 Fab density at the 3-fold spikes of the AAV9 capsid near regions HVR-V and HVR-VIII. Based on surface accessibility and previously published data, 493-TQNNN-496 and 585-SAQAQ-589 were selected for further studies. Residues 493-496 are largely conserved between AAV serotypes; as PAV9.1 was determined to be specific for AAV9, mutagenesis experiments to validate this neutralizing epitope were therefore focused around aa585-589, which is highly divergent between serotypes and more likely to be responsible for PAV9.1 binding and neutralization. Residues 585-589 in the AAV9 *cap* gene were swapped for those resembling equivalent residues in AAV2 (RGHRE), AAV3B (SSNTA), and AAV8 (QQNAA) as well as an intermediate AAV8/9 mutant (AAQAA), which only varies from the wild type AAV9 VP by 2 residues. PAV9.1 binding to the AAV8/9 intermediate mutant was moderately reduced, while PAV9.1 binding to the AAV2, 3B, and 8-based mutants was nearly completely ablated. AAQAA and RGHRE AAV9 mutants performed equivalently to wtAAV9 *in vitro*, and PAV9.1's ability to neutralize transduction was lost at all titers tested (1:1280-1:81,920), indicating at least a 64-fold reduction in neutralizing capacity for these mutants. These mutants are currently being evaluated at lower PAV9.1 titers. We are also conducting initial studies to evaluate PAV9.1 neutralization of SSNTA and QQNAA mutants, and plan to test all mutants' abilities to evade PAV9.1-mediated neutralization *in vivo*. This study supports the development of next generation vectors for use in the clinic that are able to evade specific neutralizing antibody responses.

Somatic Stem Cell Therapies

736. Early and Stable Engraftment of a Population of Highly-Enriched Long-Term Multi-Potent Hematopoietic Stem Cells

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Understanding the biology of hematopoiesis after transplant is fundamental to improving treatments such as gene therapy and gene editing. Competing models of hematopoiesis after autologous transplant have been proposed by tracking individual retrovirus-transduced CD34$^+$ cell clones in animals and patients, and suggest slow kinetics of hematopoietic stem cell (HSC) contribution. Here we followed longitudinal hematopoiesis in a total of eighteen nonhuman primates (NHP) after autologous, myeloablative transplantation of lentivirus (LV) gene modified cells. Three phases of hematologic recovery were established: initial engraftment (0-3 months), stabilization (3 months to 1 year) and homeostasis (>1 year). In ten animals, we tracked >200,000 individual clones using integration site analysis (ISA) for up to 7 years *in vivo*. Contributions of HSC-like clones were evident during all three phases of reconstitution. Simultaneous clone tracking by lentivirus DNA barcode sequencing (DBS) in two animals validated early engraftment of HSC-like clones, and highlighted an ISA bias toward clonal succession kinetics. To resolve this difference, we randomly sampled available datasets for each animal to determine whether reconstitution patterns could be changed or reverted. Random sampling validated clonal stability as the prevailing biology and revealed sampling frequency to be the most important factor in skewing of ISA data towards clonal succession. This direct comparison of clone tracking methods *in vivo* allowed us to determine recommended sampling frequencies for gene therapy studies where ISA is the only available clone tracking method. **Most importantly, these data demonstrated a subpopulation of multipotent CD34$^+$ cells which engrafts very early and contributes to multi-lineage hematopoiesis over years of recipient lifetime in the autologous myeloablative setting.** Identification of this highly HSC-enriched population would have tremendous impact in the fields of gene therapy and gene editing by reducing vector or editing reagent needs and limiting off-target effects. To identify this population, purified CD34$^+$ cells from four animals were characterized using available and cross-reactive human HSC markers. We demonstrate the phenotypic subset of CD34$^+$CD90$^+$CD45RA$^-$ cells to be the target HSC-enriched population *in vitro*. To evaluate this population *in vivo*, a competitive repopulation transplant strategy was employed in four animals. Surprisingly, we observed that this subpopulation was exclusively responsible for short-term, long-term and multi-lineage hematopoietic engraftment in all animals. Retrospective analysis of 15 transplanted animals revealed that CD34$^+$CD90$^+$CD45RA$^-$ cell doses can predict the onset of hematopoietic recovery after transplant in this model. Further characterization demonstrated that this HSC-enriched population is evolutionarily conserved in NHP and humans. These data represent a new paradigm for hematopoietic transplantation including gene therapy and gene editing.*These authors contributed equally to this work.

737. Successful Microglia Reconstitution with Allogeneic Hematopoietic Stem and Progenitor Cells Through Intracerebroventricular Injection in Mice

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Lysosomal storage disorders (LSD) are a group of rare inherited metabolic diseases caused by the defective activity of specific lysosomal hydrolases. Abnormal buildup of enzyme specific substrates leads to multiple organ dysfunction, with severe involvement of the central nervous system (CNS) in the majority of the patients. Hematopoietic cell transplantation (HCT) and/or hematopoietic stem cell (HSC) gene therapy were shown to provide therapeutic benefit in some LSDs and to alleviate CNS damage. This is due to the ability of the transplanted cells to generate tissue reservoirs of functional lysosomal enzymes consisting of reconstituted tissue-resident myeloid populations. In the attempt to enhance HSC-based therapeutic efficacy on the LSD brain, our group has implemented a novel protocol for the delivery of hematopoietic stem and progenitor cells (HSPCs) by intracerebroventricular (ICV) injection in myeloablated recipients in conjunction with standard HCT (Capotondo et al., submitted). This approach allows a faster and more extensive reconstitution of brain myeloid cells, possibly including microglia, by the progeny of the transplanted HSCPs, compared to the standard intra-venous (IV) transplantation. This results in rapid enzyme delivery to the affected brain that could benefit the CNS disease. In order to anticipate clinical transferability of these findings in current alloHCT practice, we tested the impact of ICV delivery of allogeneic HSPCs on overall survival and CNS microglia engraftment in mice undergoing HCT from mismatched donors. We tested the ICV + IV HCT in the context of MCH-mismatched (BALB/cJ CD45.2 donors into B6.SJL CD45.1 recipients) as well as minor antigen-mismatched (MHC-matched; BALB_B CD45.2 donors into B6.SJL CD45.1 recipients) transplant settings. Donor-derived HSPCs (isolated by lineage depletion from bone marrow) were labeled with a GFP lentiviral vector and subsequently delivered by ICV injection into recipient mice conditioned by lethal total body irradiation (TBI) a few hours after IV transplantation of unlabeled total bone marrow cells from the same donors. During post-transplant follow up, recipient mice survived long term, demonstrated steady increase in the body weight and similar high donor engraftment, without increased incidence of graft-vs-host disease (GvHD) as compared to IV-only mice. At sacrifice, 9 or 10 weeks post-transplant, ICV transplanted animals from the minor antigen-mismatched group showed similar sustained donor chimerism and multi-lineage differentiation in peripheral blood, bone marrow, spleen and thymus compared to controls. Interestingly, in the CNS, recipients receiving HSPCs by ICV delivery showed a dose-dependent increase in the donor chimerism within myeloid/microglia cells as compared to IV-only recipients,

thus confirming the added value of HSPC ICV transplantation for brain myeloid chimerism with the donor. Observation of transplant recipients within the MHC-mismatch setting is still on going for long-term monitoring of GvHD incidence and microglia chimerism up to 16 weeks post-transplantation. Overall, these data provided evidence of the safety and feasibility of direct delivery of allogeneic HSPCs into CNS. High level of microglia reconstitution in CNS with no obvious negative impact on survival supports the feasibility of the application of this approach in the context of allogeneic HCT for LSD patients with severe CNS involvement.

738. Novel Molecular and Functional Insight into Cyclosporine-Mediated Enhancement of Human Hematopoietic Stem Cell Gene Therapy

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Shortening ex-vivo culture and lowering vector doses remain a high priority goal for hematopoietic stem and progenitor cell (HSPC) gene therapy. We previously showed that Cyclosporine A (CsA) significantly improves lentiviral vector (LV) transduction (TD) efficiency in long-term repopulating human HSPC. We assessed here its efficacy and safety in clinical culture conditions using bone-marrow (BM)-derived HSPC and clinical-grade LV. Furthermore, we provide novel molecular insight regarding its mechanism of action identifying a novel, more potent and broadly active cyclosporine in the process. The single-hit CsA protocol yielded comparable vector copy numbers/human genome as the two-hit standard in the BM of transplanted mice long-term. Interestingly, the highest engraftment levels were observed for HSPC transduced in presence of CsA. This is potentially explained by a CsA-mediated reduction of HSPC proliferation, increase in the quiescent G_0 fraction and preservation in culture of the more primitive stem and multipotent progenitors. Importantly, no alterations in the vector integration profiles could be detected between CsA and control transduced HSPC.

While searching for host factors involved in the CsA-mediated increase in LV transduction, we verified that knock-down of cyclophilin A (CypA), a known CsA interactor and HIV co-factor, impairs LV transduction also in human HSPC. This suggests that the CsA-mediated increase in TD is likely suboptimal in HSPC due to its well documented negative impact on the CypA-LV capsid interaction. In agreement with this hypothesis, LV harboring CypA-independent capsids benefitted more from CsA and we identified a CypA-independent derivative (dCsA) that performed even better than CsA in improving TD in human and murine HSPC. dCsA yielded almost 100% TD efficiency and up to 3 vector copies/genome after a single LV hit at the moderate multiplicity of infection of 10 in the clinically relevant mobilized peripheral blood-derived HSPC, without altering their biological properties. Both cyclosporines seem to overcome basal but also type I IFN-induced LV restriction in HSPC. But differently from CsA, dCsA increased TD also in unstimulated HSPC, activated T-cells and using an integrase defective LV (IDLV). In line with improved IDLV transduction, dCsA enhanced by 2-fold also gene targeting efficiency in murine and human HSPC, particularly in the more primitive CD34+CD133+CD90+ fraction, and allowed scaling down of the IDLV dose 5-fold without compromising targeting efficiency. Overall, these TD protocols represent a potential alternative over the current clinical standard. Moreover, the identification of a more potent and broadly effective cyclosporine provides molecular clues regarding LV restriction blocks in human hematopoietic cells and represents an important step forward in the development of more efficient gene and cell therapy strategies.

739. *Ex Vivo* Blood Conditioning Using Tissue Engineered Reactors

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Human mesenchymal stromal cells (MSCs) metabolize and secrete molecular mediators that can globally shift a wound healing response. Controlled exposure to this cell therapy has been challenging with intravenous infusion of MSCs due to limits in tolerable cell dose and the rapid clearance of MSCs by the body. We have developed an ex vivo MSC technology that maintains MSC viability and enables the continuous, controlled delivery of MSC molecules into the blood stream in a clinical setting. MSCs were integrated into hollow-fiber bioreactor devices whereby the cells, separated by a permeable membrane, can directly and dynamically condition a patient's blood without entering the body. A human scale prototype of the technology will be presented showing sustained cell viability and function throughout cGMP manufacturing. Pharmacological analysis of this bioreactor technology in a large animal toxicology study allowed for an unprecedented look at MSC therapy during product use. The study verified a pharmacokinetic and pharmacodynamic response to ex vivo MSCs that is consistent with a potent immunomodulatory mechanism of action in large animals. The presentation will also report encouraging *in vivo* survival results of ex vivo blood conditioning with MSCs in a canine model of ischemic acute kidney injury (AKI). A Phase I clinical trial design and early enrollment in AKI patients will announced as well. Ex vivo blood conditioning with this reactor technology can find many other clinical applications in regenerative medicine and immunotherapy.

740. Development of an Autologous Gene-Modified Cell Therapy for the Treatment of Localized Scleroderma

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Background: Localized scleroderma is an autoimmune inflammatory fibrosing disorder involving skin and subcutaneous tissues that may cause permanent functional disability and disfigurement. Although current treatments are effective in the active stage of disease, most do not address the damage component, or fibrotic stage. FCX-013 is an

autologous, genetically-modified human dermal fibroblast (GM-HDF) cell therapy in development for the treatment of Localized Scleroderma. FCX-013 expresses interstitial collagenase (matrix metalloproteinase 1; MMP1) to address the fibrotic lesions that cause morbidity in these patients. MMP1 expression is controlled by an inducible promoter, modulated by a RheoSwitch Therapeutic System® (RTS®) gene switch and oral administration of the activator ligand veledimex. Isolated patient fibroblasts are modified with a lentivirus (LV) containing the RTS® components and the MMP1 gene (LV-MMP1), followed by amplification of the cells in culture, then cryopreservation. **Methods:** Towards development of FCX-013, GM-HDFs were generated using normal human dermal fibroblasts transduced with dilutions of research-grade LV. Transduced cells (HDF-MMP1) were passaged 3 times to allow for LV integration, and cells were assessed for integrated copy numbers and for MMP1 expression levels with and without veledimex. Similarly treated non-modified cells (non-GM-HDFs) served as a control. To address the potential efficacy of FCX-013, we assessed whether intradermal delivery of HDF-MMP1 plus oral veledimex could reverse bleomycin-induced dermal fibrosis in a mouse model, using dermal thickness measurements as a primary endpoint. Levels of MMP1 were also measured in the serum to assess systemic exposure. **Results:** HDF-MMP1 cells used for the *in vivo* study had a high integrated copy number of LV-MMP1 (5.7 copies/cell) and expressed >1500 ng/mL of total MMP1 *in vitro* in the presence of veledimex. Without veledimex, MMP1 was expressed to 15-20 ng/mL. *In vivo*, a single intradermal injection of HDF-MMP1 plus daily oral veledimex was able to significantly reverse the development of fibrotic lesions as determined by dermal thickness measurements. This treatment also significantly reduced the thickness of the underlying muscle layer. Moreover, results suggest that even low levels of MMP1 expression have a positive impact in reducing fibrosis. Finally, MMP1 was detected only in the serum of HDF-MMP1 injected mice treated with veledimex activation. **Conclusions:** The results of this proof of concept study suggest that intradermal delivery of FCX-013 in combination with oral veledimex has the potential to reverse dermal fibrosis. Localized delivery shows efficacy and minimizes systemic exposure to MMP1. Current IND-enabling pharmacology/toxicology studies are underway with clinically relevant copy numbers for dose-range testing of FCX-013 and veledimex.

741. Reprogramming Human Gallbladder Cells into Insulin-Producing Beta-Like Cells

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Gallbladder and cystic duct (GBCs) are attractive sources of potentially renewable β-like cells not only for their close developmental origin to the pancreas, but also for their amenability for surgical removal without serious health effects. Here, we report of a robust methodology for expansion culture and genetic reprogramming of primary human gallbladder cells (from multiple unrelated donors) to β cell fate for potential autologous cell therapy in type 1 diabetes. GBCs were expanded to 100 million cells after 4 passages in 1-2 months and maintained for at least 12 passages. Transcriptome profiling revealed GBCs to be deficient in factors important for beta-cell development, specification, and maintenance (NEUROG3, MAFA, NKX6-1, NKX2-2, PAX4, PAX6, NEUROD1, INSM1, RFX6, GCK, and INS). MNX1 and PDX1 were expressed at low levels in cultures. MicroRNA sequencing also revealed GBCs expressed many microRNAs not found in β cells and more importantly, GBCs lacked microRNAs associated with insulin production, secretion, β cell differentiation such as hsa-let7a-1, -miR-7, -miR-26. GBCs were reprogrammed into insulin-producing pancreatic β-like cells by a combination of adenoviral expression of hallmark pancreatic endocrine transcription factors *PDX1, MAFA, NEUROG3*, and *PAX6 in vitro*. The combination of all four transcription factors resulted in the highest insulin expression in reprogrammed GBCs (rGBCs). rGBCs also expressed high levels of hallmark β cell-associated genes *NEUROD1, NKX2-2, RFX6, PAX4, MAFB, HOPX, SST, GHRL, CHGA, TMEM27, SYP, KCNJ11, PCSK1* and *ABCC8* comparable to islet cells, although *GCG* was not induced. Retinoic acid, GLP-1, FGF10, DAPT, T3, Alk5 inhibitor, isoxazole, and B27 in the reprogramming medium significantly improved both *INS* and *NKX6-1* induction reaching 10% of primary islet cells. Average transduction efficiency was 60% yielding between 9-25% C-peptide+ cells by immunostaining. On average, we observed an 2.5-3.5-fold increase in C-peptide secretion in response to high glucose. RNA sequencing showed that rGBCs significantly upregulated key genes that are associated with β cell state while downregulating genes present in primary GBCs. Gene set enrichment analysis of the 151 most differentially expressed "Beta genes" showed that rGBCs upregulated the molecular signatures involved in the regulation of β cell development, gene expression, MODY, and Type 1 Diabetes Mellitus. Similar to rGBC transcriptome, the rGBC microRNAome resembled human ß cells. Unbiased hierarchical clustering revealed that the global microRNA contents of rGBC had a stronger correlation with β cells (R=0.851) compared to primary GBC (R=0.665). Taken together, mRNA and microRNA expression profiling and protein assays showed that rGBCs adopted an overall pancreatic β-like state. In summary, we have developed, for the first time, a reliable and robust culture expansion and genetic reprogramming of multiple unrelated patient-derived adult primary GBCs into pancreatic β-like cells *ex vivo*, thus showing that the human gallbladder is a potentially rich source of reprogrammable cells for autologous cell therapy in diabetes.

742. Intra-Cavity Neural Stem Cell/Polymer Scaffold Composite Therapy Targets Post-Operative Tumor Foci in a Novel Surgical Resection Model of Medulloblastoma

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Background: Cytotoxic neural stem cells (NSCs) are a promising treatment for Medulloblastoma (MB), the most common primary pediatric brain tumor. Surgical resection is part of the clinical standard

of care for MB, yet tumoricidal NSC therapy for post-operative MB remains unexplored. In this study, we developed an image-guided model MB surgical resection/recurrence and investigated multiple aspects of intra-cavity NSC therapy for post-operative MB. **Methods:** To create the first image-guided resection model of orthotopic MB, human MB cells (D283 and Daoy) engineered to express fluorescent and bioluminsescent optical reporters were orthotopically xenografted into the brain of Nude mice. Image-guided surgical resection was used to resect the established tumor and track recurrence. Brain-derived NSCs and 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:** Image-guided surgery reduced 92% of the MB volumes, and we discovered the rate of post-operative MB regrowth increased 3-fold compared to pre-resection growth. *In vivo*, real-time kinetic imaging showed that NSCs rapidly to MB, migrating 1.6-fold faster and 2-fold farther in the presence of tumors, and migrated through the cerebellum of mice to populate MB in the contra-lateral hemisphere. Using the resection/recurrence model, we found that seeding of cytotoxic NSCs into the post-operative surgical cavity decreased MB volumes 15-fold and extended median survival 133%. As a novel autologous therapy, we found that iNSCs homed to MB cells, while intracavity iNSC therapy suppressed post-surgical tumor growth and prolonged survival of MB-bearing mice by 123%. **Conclusions:** We report a novel image-guided model of MB resection/recurrence and provide new evidence that cytotoxic NSCs/iNSCs delivered into the surgical cavity effectively target residual MB foci, providing a new treatment option to MB patients.

743. Assessment of a Cell Therapy Approach for Duchenne Muscular Dystrophy Using Mesoangioblasts and Transposable Vectors

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Introduction: Duchenne muscular dystrophy (DMD) is a progressive, lethal muscle wasting disease that has no treatment till date. It is caused by mutations in the dystrophin gene. Autologous transplantation of genetically modified myogenic stem cells would be an attractive therapeutic option for the disease. However, there has been very modest success in cell therapy for muscular dystrophy due to the large size of the dystrophin gene and transgene silencing. Mesoangioblasts (MABs) are muscle progenitor cells with an ability to fuse with myofibers. **Aim of the study:** This study investigates the use of the PiggyBac transposon for full-length dystrophin expression in MABs in a cell therapy approach of a DMD mouse model (mdxSCID). **Methods:** DMD murine MABs were transfected with transposable expression vectors for full-length dystrophin and GFP/nlacZ, and transplanted intra-muscularly or intra-arterially into mdxSCID mice. **Results:** Intra-arterial delivery indicated that the mesoangioblasts retained their ability to cross the vessel walls, migrate to regenerating muscles and mediate dystrophin expression. By intra-muscular delivery, expression of dystrophin and dystrophin-associated proteins was restored in 18-45% of myofibers in the murine muscles. Moreover, expression was stable for the assessed period of five months after transplantation. Dystrophin protein levels in transplanted muscles were found to be between 10-30% of wild type. Furthermore, approximately 3% of the satellite cell population comprised mesoangioblast-derived cells, indicating that transplanted MABs retained their ability to colonize the satellite cell niche. Also, transposase DNA levels were at the detection limit, indicating a very low risk of further transposition events *in vivo*. The mapping of transposition sites in MAB genome indicated that approximately 70% of the integrations were in the intergenic regions (safe harbors), and no integration was observed in an exon. For functional restoration assessment, atomic force microscopy assays were performed on transplanted muscles, which showed that almost 80% of fibers showed elasticity properties restored to those of wild type muscles. Morphological assessment of transplanted muscles indicated reduction in fibrotic and scar tissue in the muscle, as well as reduction of cross section area of individual muscle fibers. **Conclusion:** These findings provide a proof-of-principle that the PiggyBac transposon system has the potential to express full-length dystrophin in dystrophic mouse muscles, and bring about morphological and functional amelioration of the dystrophic muscles. Hence, this method could contribute to improvement cell-based therapies for DMD in the future.

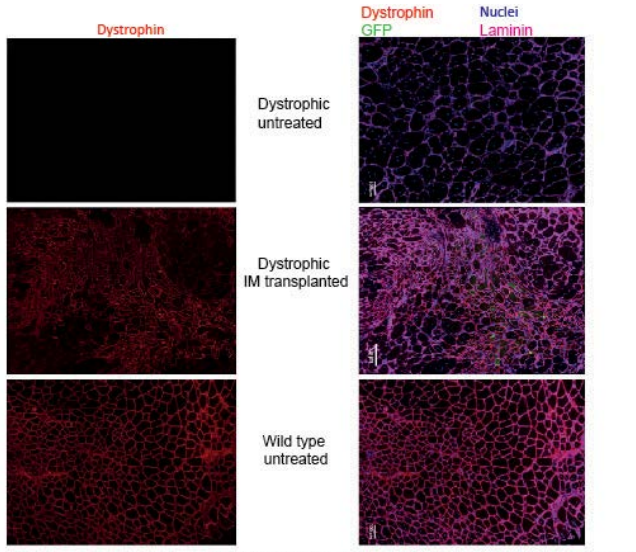

Figure 1: Immunostaining for dystrophin, laminin, GFP and DAPI. Scale bars indicate 100 µm. The centre panel in the figure indicates restoration of dystrophin expression in a TA muscle cross section by transplantation of genetically modified MABs.The top and bottom panels are dystrophic and wild type TA muscle cross sections respectively.

Ex Vivo Gene Editing

744. Genome Editing of Hematopoietic Stem Cells to Increase Fetal Hemoglobin in Sickle Cell Disease and β-Thalassemia

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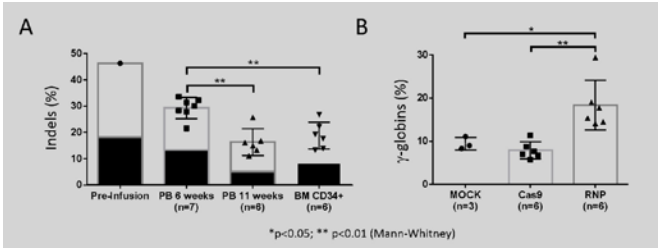
We seek to develop new therapies for sickle cell disease (SCD) and β-thalassemia, severe anemias caused by *HBB* (β-globin) gene mutations that usually become symptomatic after birth as red blood cell (RBC) hemoglobin (Hb) switches from fetal (HbF, $\alpha_2\gamma_2$) to adult (HbA, $\alpha_2\beta_2$). Hereditary persistence of fetal hemoglobin (HPFH) is a benign condition in which HbF exceeds 20% of total Hb in adult RBCs and the clinical manifestations of co-inherited SCD or β-thalassemia mutations are largely suppressed. Previously, we used CRISPR/Cas9-mediated non-homologous end joining (NHEJ) to recapitulate HPFH caused by a 13-nt deletion in the *HBG1* (γ-globin) gene promoter. Disruption of this region in human CD34$^+$ cells raised HbF to potentially therapeutic levels in RBC progeny generated by in vitro differentiation (Nat Med 22, 2016). Now, we are investigating whether this region can be disrupted in HSCs. We electroporated Cas9 or Cas9/single guide (sg) RNA ribonucleoprotein complex (RNP) into normal mobilized CD34$^+$ cells and cultured them with SCF, FLT3-L, and TPO. At 4 days, viability was ~90% for non-electroporated cells (MOCK) and those electroporated with Cas9 or RNP. In RNP-treated cells, 46% of γ-globin alleles contained indels with ~40% of those being the 13-nt HPFH deletion. 10^6 cells from each group (MOCK, n=4; Cas9, n=7; RNP, n=7) were transplanted into busulfan-conditioned immunodeficient (NSG) mice. At 6 and 11 weeks, the proportion of CD45$^+$ human cells in peripheral blood (PB) ranged from 21-36% with no significant differences between groups. At 16-18 weeks, human cell engraftment in bone marrow (BM) was: MOCK, 27±10%; Cas9, 61±15%; and RNP, 42±11% (Mean±SD). The indel frequency in the RNP group was: PB, 6 weeks; 29±4%; PB 11 weeks, 16±5%; BM 16-18 weeks, 19±5% (Figure 1A). We isolated human CD34$^+$ cells from mouse BM at 16-18 weeks post-transplant and cultured them to generate erythroid cells. Edited and control populations exhibited no detectable differences in erythroid maturation assessed by flow cytometry markers and cell morphology. HPLC analysis for %HbF in late stage erythroblasts and reticulocytes at day 15 showed: MOCK, 9±2%; Cas9, 8±2%; and RNP, 18±6% (Figure 1B), representing an approximate doubling in the edited population ($p<0.05$). The magnitude of HbF increase is likely substantially greater in RBCs derived from edited CD34$^+$ cells, which comprised only 20% of the starting population. Use of NHEJ in HSCs to increase HbF in adult RBC progeny has several potential advantages for treating SCD and β-thalassemia. NHEJ occurs more efficiently in HSCs compared to homology directed repair for correcting specific mutations. Moreover, the same NHEJ strategy can be used to treat hemoglobinopathies caused by different mutations. Our findings bring us one step closer to this therapy by showing the possibility to recapitulate HPFH in human HSCs. Future studies will focus on confirming our initial findings and optimizing the editing process in HSCs.



(A) Indels determined by TIDE in CD34$^+$ cells pre-xenotransplantation and at indicated times afterward in peripheral blood (PB) and bone marrow (BM) CD34$^+$ cells (Mean±SD). The 13-nt HPFH deletion is represented in black. (B) CD34$^+$ cells from mouse bone marrow (BM) 16-18 weeks after xenotransplantation were cultured for 15 days to generate late stage erythroid precursors. Graph shows the %γ-globin [(Gγ+Aγ)/(Gγ+Aγ+β)] measured by HPLC.

745. Multiplexing CRISPR-Cas9 Genome Editing in Human Hematopoietic Stem and Effector Cells

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Precise and efficient manipulation of genes (genome editing) is crucial for understanding the molecular mechanisms that govern human hematopoiesis and for developing novel therapies for diseases of the blood and immune system. However, simultaneous manipulation of multiple genomic loci using a site-specific programmable CRISPR/Cas9 endonuclease system is particularly challenging and has not been successfully demonstrated in primary hematopoietic cells. In this work we show that we can edit multiple genes (CCR5, HBB, IL2RG, ASXL1, RUNX1) by homologous recombination simultaneously in primary hematopoietic stem and progenitor cells (HSPCs) as well as human effector T-cells. By combining rAAV6 DNA donor delivery, the CRISPR/Cas9 system delivered as ribonucleoproteins, and a FACS-based enrichment scheme for cells with multiplexed targeted integration events, we can simultaneously create up to 8 precise genetic changes in these therapeutically relevant primary cell types. We are able to achieve efficiencies up to 30%, 11%, 4% and 1% for monogenic, di-genic, tri-genic and tetra-genic targeting in HSPCs, respectively. In vitro methylcellulose colony formation assays showed mixed, myeloid and erythroid colony forming potential in multiplexed targeted HSPCs. More importantly, transplantation studies in immunodeficient NSG mice showed multiplexed targeting in HSPCs with long-term engraftment potential. It was not too long ago that 1-2% targeting efficiencies at one gene in human HSPCs were state-of-the art findings and now we can achieve those rates at four genes simultaneously. Collectively, these findings raise genome editing to a whole new level in terms of both using it as a research tool to genetically interrogate complex pathways and as a potential method to cure human disease of hematopoietic origin.

746. AAVHSC Vectors Mediate Highly Precise and Efficient Homologous Recombination-Based Gene Editing

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The permanent correction of pathogenic mutations by genome editing is a viable and attractive therapeutic strategy. To date, most editing platforms utilize the induction of nuclease-mediated DNA breaks of the genome as a first step in initiating the editing events. These breaks are then primarily repaired using the error-prone non-homologous end joining (NHEJ) pathway. The presence of a donor DNA correction template at the site of the DNA break, can result in a minor fraction of the breaks being repaired by the more precise homologous recombination (HR) pathway. However, NHEJ-mediated repair of the majority of nuclease-mediated breaks result in insertion/deletion errors at the site of breaks or inclusion of the entire vector genome including the inverted terminal repeats (ITRs), in both orientations via NHEJ. The off-target effects of nuclease-based DNA cleavage further pose unknown mutagenic risks. Thus, a precise, efficient and predictable genome editing technology based solely on HR pathways would represent a significant advance in the field. We recently described a group of novel natural AAVs isolated from CD34+ human hematopoietic stem cells known as AAVHSCs. Here we evaluated the capacity of AAVHSC editing vectors to mediate precise and efficient genome editing in vitro and in vivo. Editing vectors were designed to insert a promoterless GFP reporter cassette into the human PPP1R12C gene located in the safe harbor site, AAVS1, such that expression would be driven by the chromosomal PPP1R12C promoter. AAVHSC editing vector genomes included homology arms that flanked the promoterless reporter and specified the target genomic site to be edited. Editing was measured by GFP expression at the cellular level and confirmed by multiple molecular assays including Sanger and Next Generation sequencing. Phenotypic editing efficiencies up to >50%, were observed by flow cytometry for GFP expression in primary cells including human CD34+, hepatic sinusoidal endothelial cells and myotubes and myoblasts. Editing efficiencies of AAVHSC vectors were significantly higher than AAV2, AAV6 and AAV8, approximately 10-50-fold, *in vitro*. Sequence analysis of edited genomes revealed no evidence of indels or inclusion of AAV ITRs, hallmarks of the utilization of the NHEJ pathway. BRCA2, an essential mediator of HR, was found to be critical for successful editing, indicating that HR was the underlying operative mechanism. In depth unbiased on and off target analyses revealed that editing was highly precise (Wright et al, ASGCT 2017). Similar editing outcomes were observed at other genomic loci. We additionally tested the capacity of AAVHSC vectors to mediate in vivo targeted insertion of a promoterless luciferase gene into the murine safe harbor locus Rosa26, such that expression could only occur following accurate insertion downstream from the Rosa26 promoter. Intravenous injection of AAVHSC Rosa26 editing vectors led to rapid and sustained widespread expression of luciferase with no apparent toxicity. Mice injected with control AAVHSC vectors without homology arms did not express luciferase, confirming that expression resulted from accurate editing. Significantly, our results demonstrate that AAVHSC represent a uniquely HR-based, highly precise and efficient gene editing modality in a single component, nuclease-free platform with a built-in delivery component. This now enables the development of therapeutic in vivo genome editing for the treatment and potential cure of human diseases.

747. Correction of SCID-X1 by Targeted Genome Editing of Hematopoietic Stem/Progenitor Cells (HSPC) in a Humanized Mouse Model

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Targeted genome editing by engineered nucleases has brought the goal of gene correction within the reach of gene therapy. A candidate disease for HSPC gene correction is SCID-X1, because gene therapy trials with integrating vectors showed robust clinical efficacy even from few corrected cells but also the occurrence of leukemias due to insertional mutagenesis and unregulated transgene expression. To support the rationale and explore the safety of gene correction we developed a mouse model carrying a mutated human *IL2RG* gene in place of the mouse *Il2rg*, allowing use of the same reagents developed for gene correcting human cells. To evaluate efficacy and safety of hematopoietic reconstitution from a limited number of corrected HSPC we performed competitive transplants with WT and *IL2RG-/-* HSPC and found that 10% of WT cells fully reconstitute the lymphoid compartments and that administration of a conditioning regimen before HSPC infusion is required to protect against the risk of lymphoma development from the transplanted progenitors. To demonstrate functional correction of the disease phenotype in mice following *ex vivo* gene correction we developed a gene editing protocol for mouse HSPC. We first tested gene correction of Lin- HSPC by the delivery of donor DNA template by IDLV followed by electroporation of ZFN mRNAs. This protocol yielded high on-target nuclease activity (40%) and a mean of 6% transgene integration by HDR but also high cytotoxicity (65% cell loss) under the conditions we used. Measuring the percentage of edited cells (either by NHEJ or HDR) long-term within the HSC compartment of transplanted mice, we found that it was nearly undetectable, possibly due to the sensitivity of mouse HSC to *ex vivo* manipulation. Therefore, we developed a new strategy exploiting LV-delivery of CRISPR/Cas9 technology, which enabled substantial levels of targeted DNA repair by NHEJ (up to 70%) and HDR (up to 25%) with minimal cytotoxicity. Upon in vivo transplant, we were able to demonstrate long-term engraftment of the edited HSC. Importantly, the gene corrected cells were able to generate lymphoid lineages (B and T cells), showing a clear selective advantage over the un-corrected SCID cells *in vivo*. These data demonstrate functional correction of the defective *IL2RG* gene by targeted editing. Furthermore, upon challenging the mice with a murine pathogen we observed viral-specific γIFN production

by CD8+ gene corrected cells, proving their in vivo functionality. The corrected cells persisted long-term in the mice indicating successful editing of at least 1% HSPC able to sustain long-term lymphopoiesis and partially correct the disease phenotype. These results established the key parameters underlying safe and effective rescue of the disease phenotype and provide stringent *in vivo* validation of the gene editing strategy and thus will be crucial for the design of a protocol for the clinical testing of SCID-X1 gene correction.

748. Nuclease-Free AAV-Mediated *Il2rg* Gene Editing in X-SCID Mice

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X-linked severe combined immunodeficiency (X-SCID) has been successfully treated by hematopoietic stem cell (HSC) transduction with retroviral vectors expressing the interleukin 2 receptor subunit gamma gene (*IL2RG*), which is required for normal T cell development and homeostasis. Unfortunately, several of these patients developed malignancies due to vector integration near cellular oncogenes, highlighting the risks of insertional mutagenesis. This adverse side effect could in principle be avoided by accurate *IL2RG* gene editing with a vector that does not contain a functional promoter or *IL2RG* gene. Here we show that nuclease-free adeno-associated virus (AAV) gene editing vectors packaged in serotype 6 capsids can insert a partial *Il2rg* cDNA at the endogenous *Il2rg* locus in X-SCID murine bone marrow cells, and that these edited cells repopulate transplant recipients and produce $CD4^+$ and $CD8^+$ T cells.

We constructed an AAV6 vector homologous to the deleted *Il2rg* locus in X-SCID mice, but containing a partial *Il2rg* cDNA at exon 3 (AAV-Il2rg3-8). AAV-Il2rg3-8 does not include the initiation codon or promotor, so random integration events will not lead to Il2rg expression, but homologous recombination at the endogenous locus creates a complete *Il2rg* reading frame expressed from the *Il2rg* promotor. X-SCID mouse bone marrow cells were infected overnight with AAV-Il2rg3-8 and delivered by IV injection into irradiated X-SCID recipients. Circulating, edited T cells increased over time and persisted for more than 8 months, and expressed physiological levels of Il2rg protein. AAV-Il2rg3-8 edited cells constituted ~10% of the donor peripheral white blood cells, and a higher proportion of their $CD3^+$ splenocytes (>58% of donor cells), whereas edited BM cells and $CD3^{neg}$ splenocytes were present at lower levels (<1%). qPCR-based editing frequencies correlated with the frequency of $CD3^+$ cells in peripheral blood, bone marrow and spleens of transplanted mice. Accurate editing was confirmed by sequencing genomic DNA and cDNA samples from treated mice. Although random integration was detectable, it was extremely rare (< 0.4%), despite the fact that the majority of infected BM cells were capable of transiently expressing GFP from an AAV6 vector. The T cell receptor repertoire of $CD3^+$ splenocytes in AAV-Il2rg3-8-treated mice was similar to that of wild-type mice, and significantly more diverse than that of X-SCID controls. Secondary transplant recipients also developed increasing numbers of edited lymphocytes in the blood and spleen, including IgD+ B cells, demonstrating successful editing in long-term repopulating cells. None of the transplant recipients developed malignancies. Although several groups have reported the use of site-specific nucleases to stimulate gene editing in hematopoietic cells, including editing of the human *IL2RG* locus, our results demonstrate that therapeutically relevant HSC gene editing frequencies can be achieved by AAV vectors in the absence of site-specific nucleases, and suggest that this may be a safe and effective therapy for hematopoietic diseases where in vivo selection can increase edited cell numbers.

749. Correction of X-Linked Severe Combined Immunodeficiency in Human Hematopoietic Stem and Progenitor Cells

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X-linked Severe Combined Immunodeficiency (SCID-X1) is a monogenic disorder that manifests in the first few months of life as frequent viral and bacterial infections. Pathogenic mutations in the IL2RG gene lead to defects in both the innate and the adaptive immune system. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the only curative treatment if performed in first months of life. Graft-versus-host disease, long-term immunosuppression and the inability to find matched donors are all hallmarks of allo-HSCT therapy. To circumvent these limitations, we have developed a CRISPR/Cas9-based genome-editing platform, for achieving functional correction of SCID-X1 disorder in human hematopoietic stem and progenitor cells (HSPCs). Our genome editing scheme combines Cas9 protein with the IL2RG chemically modified sgRNA to generate precise double strand breaks in HSPCs genome, at levels > 80% and as early as 48 hours, as measured by insertion and deletion (INDELs) rates. Using a homologous recombination (HR)-based genome targeting approach, we introduce a functional IL2RG cDNA, delivered via adeno-associated viral vector of serotype 6 (AAV-6), into the endogenous locus in HSPCs genome, at rates with a median of 45% (s.e.m ± 3.3). *In vitro* studies, on the functionality of the genome modified T-cells purified from healthy donors, show equivalent STAT5 signaling levels as compared to unmodified T-cells, normal activation induced proliferation response and equivalent responses to CD3/CD28 stimulation as to co-cultured with allogeneic cells. Multi-lineage potency of the IL2RG targeted HSPCs is demonstrated, *in vitro*, using limiting dilutions experiments on OP9 stromal cells transduced with a doxycycline inducible delta-like ligand 1(dll1). OP9-dll1 system is supplemented with a cytokine cocktail supporting multi-lineage differentiation. In the absence of induction, lineage differentiation of HSPCs is restricted to B- and myeloid. Dox-dependent expression of dll1 provides permissive growth conditions for both T and NK cells. Progenitor cells with markers of lymphoid, erythroid and myeloid lineages were generated at similar levels, by both IL2RG-targeted HSPCs and unmodified, mock treated

cells. *In vivo* xenotransplantation assays, with IL2RG-targeted HSPCs into immunodeficient NSG newborn mice, averaged 37% (s.e.m ± 8.7) and 24% (s.e.m ± 6.8), in the bone marrow, at week 8 and 12, respectively. Of the engrafted IL2RG-targeted HSPCs, 54% (s.e.m ± 9.4) and 49% (s.e.m ± 6.6), as assessed at week 8 and 12, respectively, comprised the targeted functional IL2RG cDNA. Circulating IL2RG-targeted cells were detected in the peripheral blood, at week 8 and 12, with a mean of 3.7% (s.e.m ± 0.6) and 4.5% (s.e.m ± 0.9), respectively. Engraftment levels of IL2RG-targeted HSPCs into immunodeficient 8-week old NSG mice averaged 79% (s.e.m ± 3.7) in the bone marrow, by week 8, with an average of 37% (s.e.m ± 6.6) of cells expressing the targeted IL2RG cDNA. Our preclinical data, thus far, demonstrates a robust, precise and efficient next generation gene therapy treatment for SCID-X1 disorder.

750. Ex Vivo Protein Replacement Using Homology Driven Genome Editing in Human B Cells by Combining Zinc Finger Nuclease mRNA and AAV6 Donor Delivery

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Adoptive cell therapies are a powerful means to introduce modified cells into patients to treat a number of diseases. Adoptive cell therapies have primarily exploited T cells, with use of B cells lagging behind likely due to the challenges associated with modification of B cells and long term culturing/differentiation methods. Harnessing B-cells by targeted gene disruption and gene addition holds promise for *ex vivo* protein replacement, and site-specific modification of B cells at distinct loci could enhance B-cell function, differentiation into plasmablasts and engraftment capabilities. Targeted insertion of transgenes could harness plasma cells' ability to produce large amounts of antibodies to become protein production reservoirs. Here we describe the development of methods to edit primary human CD19-positive B cells at distinct sites in the genome by non-homologous end joining (NHEJ) and homology-directed repair (HDR). CD19-positive B cells differentiated in vitro to plasmablasts and plasma cells produce upwards of 10,000 ng/mL of antibodies (IgG, IgM, IgA). Using electroporation of ZFN mRNA upwards of 80% modification was achieved at multiple loci, including the AAVS1, CCR5 and TRAC loci. This approach using ZFNs to modify the genome provides a means to efficiently disrupt gene expression in B cells. Transduction of CD19+ positive B cells with AAV demonstrated AAV6 superiority compared to other serotypes including AAV2, AAV5, AAV8 and AAV9 during the differentiation process to plasmablasts and plasma cells. Insertion of a transgene of interest at the AAVS1, CCR5 and TRAC loci was achieved by electroporation of ZFN mRNA together with an AAV6 transgene donor cassette containing homology arms. High levels of transgene addition were measured at all loci in the range of 38-50%. To determine what repair pathway was used to drive targeted gene addition we compared donors with matching homology arms to the nuclease target site to donors with non-matching homology arms. Donors with matching homology arms were capable of inserting at the target locus whereas non-matching homology arm donors were not, suggesting targeted gene addition occurs primarily through HDR over NHEJ in B-cells. Compared to a ubiquitous promoter, a B-cell specific promoter was capable of producing higher transgene levels as the B-cell progressed to plasmablast and plasma cells likely reflecting the redirection of B-cell activities during maturation to a plasmablast. The ability to perform genome editing on B-cells both by targeted gene disruption and gene addition holds promise for utilization of B-cells in adoptive cell therapies and the expression of therapeutic transgenes.

751. Nuclease-Targeted Gene-Editing of FOXP3 in Primary CD4+ T Cells to Generate Stable and Functional Engineered T_{reg} Products

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Regulatory T cells (T_{reg}) play a critical role in maintaining immune tolerance and preventing autoimmune disorders such as graft vs. host disease (GVHD) and Type I diabetes (T1D). Clinical approaches to improve Treg function or abundance have shown some efficacy in slowing the course of T1D. One approach, expansion and re-infusion of autologous natural Treg (nT_{reg}) has been shown to be safe, but is limited in efficacy by challenges including slow rates of *ex vivo* expansion, potential instability of nT_{reg} *in vivo* and/or the low frequency of nT_{reg} with relevant antigen-specificity. Additionally, nT_{reg} in T1D and patients with other autoimmune disorders may be functionally impaired. We are developing strategies to allow efficient generation of engineered T_{reg} (eT_{reg}) with a range of functional attributes from bulk human peripheral blood CD4+ T cells (T_{eff}). In the current study, we used a homology-directed repair (HDR)-mediated gene editing approach to generate T cells stably expressing high levels of Forkhead box P3 (FOXP3), the key transcription factor governing Treg development and maintenance. Using FOXP3-specific TALEN nucleases and AAV-based donor templates, we introduced a constitutive promoter (MND) upstream of the endogenous FOXP3 coding region in primary human CD4+ T cells. Selectable markers such as GFP, EGFRt and other gene-cassettes were built into the template to be translated in-frame upstream of FOXP3, and allowed efficient enrichment of the edited population. Despite having DNA methylation at sites that epigenetically silence the FOXP3 locus in non-T_{reg}, our strategy resulted in eT_{reg} with high levels of FOXP3 expression driving a T_{reg} phenotype, including surface markers ($CD25^{high}$, $CD127^{low}$, $CTLA4^{high}$, and $LAG3^{high}$), reduction of cytokine production (IL-2, IL-17, and IFN-γ), and suppression of stimulated T effectors (T_{eff}) in vitro. Detailed RNAseq studies also demonstrated a gene expression program most similar to cultured nT_{reg}. When comparing eT_{reg} with T cells transduced with FOXP3-expressing lentivirus (LV), eT_{reg} were found to be more phenotypically homogeneous, and expressed higher levels of FOXP3 and more uniform levels of relevant T_{reg} surface markers. As a proof-of-concept that eT_{reg} can suppress a polyclonal, autoreactive immune response, we co-infused eTreg with autologous T_{eff} into NOD-scid-Il2rgnull (NSG)

mice, and found a significant delay in GVHD in vivo compared to T_{eff} infusion alone, or mock-edited T cell co-infusion. At 50 days post-infusion, eT_{reg} were present in spleen, lung, liver, peripheral blood, and bone marrow. In contrast with T_{eff} cells, long-term engrafted eT_{reg} made IL-10 and IL-4, but lacked IFNγ and IL-2 production. These results indicated that our gene editing approach reprogramed previously non-T_{reg} committed T cells into stable FOXP3 expressing cells with immunosuppressive activity. We next generated eT_{reg} from human and mouse antigen-specific T cells with the goal of producing stable, functionally active antigen-specific eT_{reg} for treating T1D and other candidate autoimmune disorders. Together, our combined findings demonstrate efficient methods to generate eT_{reg} resulting in cell products with stable long-term FOXP3 expression and activity *in vivo*; as well as the potential to move this platform into generation of antigen-specific eT_{reg} for a range of possible future clinical applications.

Gene and Cell Therapies for Hematologic and Immunologic Diseases II

752. HSPC Mobilization in Association with *In Vivo* Foamy Virus Vector Delivery of Common Gamma Chain Promote Rapid Immune Reconstitution in the SCID-X1 Canine Model

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X-linked severe combined immunodeficiency disease (SCID-X1) is caused by null mutation in the interleukin-2 receptor gamma chain (gC) gene, *IL2RG*. This disease is characterized by abnormal development of lymphocytes and natural killer cells, lack of T-cell mediated immune responses, and low immunoglobulin levels. Fatality from infections often occurs during infancy unless affected patients receive allogeneic hematopoietic cell transplantation (HCT). HCT from an HLA-matched sibling donor can be curative but is of limited availability, while HCT from mismatched and unrelated donors is associated with decreased survivability due to graft versus host disease (GVHD). *Ex vivo* gene therapy clinical trials, in which a patient's own hematopoietic stem and progenitor cells (HSPCs) are treated and returned, are showing promising results but require expensive GMP cell processing facilities and genotoxic conditioning regimens to achieve engraftment of gene-corrected cells. With these limitations in mind, we explored *in vivo* gene therapy as a treatment for SCID-X1 using the canine model. We previously demonstrated that *in vivo* foamy virus (FV) vectors delivery of the *IL2RG* gene, driven by the human elongation factor-1 alpha promoter (FV-EF1a-gC) resulted in robust proliferation of corrected T-lymphocytes in SCID-X1 pups. However, the treated animals suffered from recurring infections likely due to incomplete and sub-therapeutic levels of immune reconstitution. In the current study, we have significantly improved our in vivo gene therapy platform by delivering a FV vector incorporating a slightly stronger enhancer-less promoter, human phosphoglycerate kinase (FV-PGK-gC), in association with administration of G-CSF and AMD3100 to mobilize HSPCs prior to *in vivo* FV vector injection. Mobilization of SCID-X1 canine newborns resulted in a 6-fold increase in circulating CD34+ HSPCs as compared to un-mobilized normal littermates. FV vector delivery into these mobilized animals demonstrated improved early and sustained kinetics of CD3+ lymphocyte expansion, comparable to T cell reconstitution observed in human *ex vivo* clinical trials. This approach also promoted thymopoiesis as demonstrated by T-cell receptor excision circles assay and resulted in greater polyclonality of immune reconstitution as measured by retroviral integration site analysis. Gene-corrected T-lymphocytes exhibited a normal CD4/CD8 ratio and a broad T-cell receptor repertoire, and restoration of gC-dependent cytokine mediated pSTAT phosphorylation. Taken together, our results suggest that this improved *in vivo* FV delivery platform (using the combination of HSPC mobilization and in vivo FV vector delivery) may provide remarkable potential for an accessible, portable and clinically translatable therapy for newborns with SCID-X1 and, possibly, for other genetic immune diseases

753. LMO2 Associated Clonal T Cell Proliferation 15 Years After Gamma-Retrovirus Mediated Gene Therapy for SCIDX1

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Fifteen years ago, we showed correction of X-linked severe combined immunodeficiency [SCID-X1, also known as γ chain (γc) deficiency] by gamma-retrovirus-mediated γc gene transfer into autologous

CD34 bone marrow cells. We previously reported that, while 9 of 10 patients were successfully treated, 4 patients developed T cell leukemia 31-68 months after gene therapy, due to insertional oncogenesis with integration of the gamma-retroviral vector near proto-oncogenes (LIM domain-only 2 (LMO2), BMI1 or CCND2). Chemotherapy failed for one patient, but was successful for all other patients, who continued to benefit from the gene therapy. These severe adverse events (SAEs) led to extensive exploration of retrovirus integration profiles and to the development of a new generation of safer, self-inactivated, vectors that have now been used in several clinical trials without any SAEs. Here we report data on a new LMO2-associated clonal T cell proliferation in one patient, 15 years after gamma-retroviral gene therapy. This T cell lymphoma was diagnosed after detection of subcutaneous nodules and a mediastinal mass, while the patient was perfectly well 3 months ahead. The lymphoma was characterized by a immature T cell phenotype (CD1a+ CD2+ CD3partial CD5+ CD7+ cTdT+ CD99+) and associated with 12% medullary infiltration (morphologically) and pleural effusion. Chemotherapy was initiated according to the EURO LB protocol. Preliminary clinical follow up shows that the patient is good responding to the treatment. The therapeutic vector was present in the blast cells, and integration site (IS) analysis (using our new optimized pipeline, INSPIIRED) disclosed expansion of a clone with vector insertion at 30kb from the LMO2 gene transcription start site, associated with upregulated expression. Additional genetic abnormalities in the patients' blast cells included Notch1 gain-of-function mutations, and CGH array analysis also revealed copy number changes such as deletion of the CDKN2A tumor suppressor gene. This late severe adverse event showed that the genetic network that controls growth in T cell progenitors can take many years to become disregulated. We will provide an update on the follow up of the patients from this trial, who are regularly monitored clinically, biologically with thymic imaging plus IS analysis.

754. Delivery and Tracking of *CCR5*-Disrupted, HIVCAR T Cells in SHIV-Infected, Non-Human Primates

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Approximately 37 million individuals worldwide are HIV-positive, ~17 million of whom are currently being treated with ART (antiretroviral therapy). Because of a persistent "latent reservoir" of HIV-infected cells, current ART is not curative. We recently showed that human T cells expressing novel anti-HIV chimeric antigen receptors (HIVCARs) based on patient-derived broadly neutralizing antibodies (bNAbs) can selectively clear HIV-infected cells *in vitro*. In current work, we have begun to test the activity of these HIVCAR T cells in a pigtail macaque (*Macaca nemestrina*) model of SHIV infection using a chimeric Simian/Human Immunodeficiency Virus (SHIV) that contains an HIV envelope with CCR5 tropism; CCR5 is the major co-receptor of HIV on $CD4^+$ T cells. Our previous work in this model demonstrates reproducible infection patterns and a lack of spontaneous control of the virus. For our HIVCAR T cell studies, we first optimized clinically relevant methods for transduction, electroporation, and expansion of modified macaque $CD3^+$ T cells. In parallel, we demonstrated activation of candidate HIVCARs in response to surface expression of the HIV envelope expressed in our SHIV. Two unique bNAb-derived HIVCARs, VRC07-523 HIVCAR and PGT128 HIVCAR, were delivered via *ex vivo* lentiviral (LV) transduction to peripheral blood $CD3^+$cells derived from a pre-SHIV macaque apheresis product. Importantly, both VRC07-523 HIVCAR and PGT128 HIVCAR target distinct epitopes on the HIV envelope glycoprotein. Each HIVCAR was stably expressed in ~75% of cells after 7-9 days in culture. In order to protect CAR T cells from infection, CCR5 was disrupted using a CCR5 megaTAL nuclease fused with Trex2, delivered via mRNA transfection immediately prior to LV transduction. Nuclease delivery resulted in *CCR5* disruption of 30-60% of the total donor $CD3^+$ cells. After demonstrating that *CCR5*-disrupted $HIVCAR^+$ NHP $CD3^+$ cells were activated against HIV-infected cells in vitro, ~10^8 autologous T cells per kg were re-infused into the macaque 14 days post-SHIV infection. More than two weeks after infusion, HIVCAR T cells were detectable in peripheral blood and tissues. Our preliminary observations indicate that HIVCAR treatment in pigtail macaques is safe and that CAR T cells can home to sites of viral replication and persist without an adverse reaction. Our combined findings establish a platform to assess engraftment, homing, and activity of novel CCR5-disrupted, HIVCAR T cells in a clinically relevant large animal model of acute and latent HIV infection.

755. First Evidence of Engraftment and Repopulation Advantage of Gene-Corrected Hematopoietic Precursors in Non-Conditioned Fanconi Anemia Patients

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Fanconi anemia (FA) is the most frequent inherited syndrome associated with bone marrow failure (BMF). As a first step in the development of a gene therapy trial in FA-A patients, we aimed at investigating the efficacy and safety of using filgrastim (12 μg/Kg/12 hours) and plerixafor (mozobil; 240 μg/Kg body weight/day) for the collection of clinically relevant numbers of mobilized $CD34^+$ cells from FA patients. While the treatment of two patients aged 15 and 16

years did not mobilize the threshold number of $CD34^+$ cells (5 $CD34^+$ cells/μL), a significant number of $CD34^+$ cells was mobilized (10 to 70 $CD34^+$ cells/μL) in each of the six FA patients aged 3-6 years. On average, 5 million $CD34^+$ cells/Kg were collected from these patients in 2-3 aphereses. After purification, a mean recovery of 45% of $CD34^+$ cells was obtained. In none of the mobilized patients severe adverse events associated with the protocol have been observed. Three of these FA-A patients have been infused with gene-corrected $CD34^+$ cells in the absence of any conditioning regimen, due to the expected proliferative advantage of corrected HSCs. In the first patient, fresh immunoselected $CD34^+$ cells were transduced for a short period of time (<36h) with the therapeutic PGK-FANCA.Wpre* lentiviral vector. In the other two patients, $CD34^+$ cells were cryopreserved for almost 2 years, until the patients demonstrated progressive evidence of BMF. Samples were then thawed and transduced under identical conditions as performed with fresh cells. Infused cell products contained 0.5 to 1.4 million $CD34^+$ cells/Kg, and vector copy numbers per cell (VCN/cell) ranged between 0.17 to 0.45. The infusion of the cell product harboring the lowest VCN/cell was associated with the detection of gene-marked cells in the PB at ratios around 0.001 VCN/cell since the first two weeks post-infusion. In this patient no further increases in the proportion of gene-marked cells could be observed in PB along the 8 months of follow-up. The infusion of $CD34^+$ cells transduced at the highest efficacy (0.45 copies/cell) conferred, however, a progressive increase of gene-marked cells in PB, that reached a proportion around 0.1 VCN/cell at the most recent follow-up (9 months post-infusion), with evidence of gene marking in both myeloid and lymphoid lineages. Bone marrow cells obtained at six months post-infusion showed a similar proportion of gene marking, and evidenced a significant phenotypic correction of the hematopoietic progenitor cells, deduced from the acquired resistance of the colony forming cells to mitomycin C (15% of BM CFCs survived to 10 nM MMC). In the third patient, the presence of gene marked cells has been recently confirmed in PB (VCNs/cell around 0.001), 2 and 4 weeks post-infusion (last follow-up). Our results demonstrate for the first time the engraftment and proliferation advantage of gene-corrected hematopoietic precursors in a FA patient. The long-term follow up of patients included in this protocol will indicate the feasibility of restoring the hematopoietic function of FA patients by means of a gene therapy approach in the absence of conditioning.

756. STAT3 Inhibition Unleashes TLR9-Induced B Cell Lymphoma Immunogenicity and T Cell-Dependent Antitumor Immune Responses

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Non-Hodgkin lymphoma (NHL) is the 6th most common cancer in the US with over 85% of tumors being of B cell origin. Growing evidence links the aggressiveness of B-cell NHL, and especially the activated B-cell-like type diffuse large B-cell lymphomas (ABC-DLBCL), to signaling by the signal transducer and activator of transcription 3 (STAT3). Mutations of upstream STAT3 regulators, such as Toll-like receptor 9 (TLR9)/MyD88 complex, are common in the ABC-DLBCL. The auto-/paracrine stimulation activates STAT3 in lymphoma cells and in tumor-associate myeloid cells, thereby promoting survival and immune evasion. To overcome the difficulty in targeting STAT3, we previously developed a strategy for cell-selective delivery of a high-affinity STAT3 decoy oligodeoxynucleotide (STAT3dODN) into TLR9+ myeloid cells and B cells. The modified CpG(B)-STAT3dODN design, based on the clinically-relevant TLR9 agonist (CpG7909), inhibited of STAT3 DNA binding and expression of downstream *MYC* and *BCL2L1* genes in human and mouse B cell lymphoma cells. We further demonstrated that IV injections of CpG-STAT3dODN inhibited growth of human Ly3 lymphoma in immunodeficient mice. In immunocompetent mice, CpG-STAT3dODN treatment induced complete regression of the syngeneic A20 lymphoma resulting in long-term animal survival. This therapeutic effect resulted from the increased immunogenicity of lymphoma cells that correlated with generation of tumor-specific and CD8/CD4-dependent T cell immunity protecting mice from tumor rechallenge. CpG-STAT3dODN injections increased CD8 to regulatory T cell ratio, while reducing expression of PD1 on CD8 T cells in dose-dependent manner. Neither CpG-scrODN nor CpG ODN alone treatments had significant effects. These results are consistent with the role of TLR9 signaling in the B cell activation, antigen-presentation and the induction for Th1-like responses. Thus, we expect that our studies will produce a technology platform for treating B-cell NHL as a monotherapy and/or combined with adoptive T-cell-based strategies.

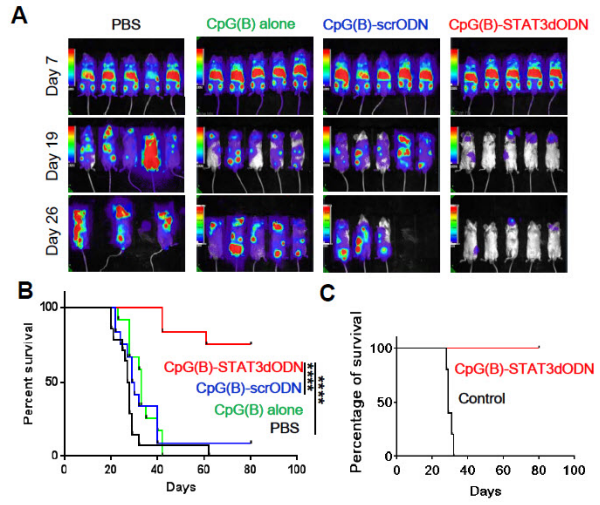

Figure 1. Therapeutic effect of CpG(B)-STAT3dODN against A20 B cell lymphoma is immune-mediated. (A, B) BALB/c mice were injected i.v. using A20 B cell lymphoma. After tumors were established (day 7), mice were treated using every other day i.v. injections of 5 mg/kg of CpG(B)-STAT3dODN, CpG(B)-scrODN, CpG(B) alone or PBS. Shown are results of bioluminescent imaging over the initial 3 weeks (A) and the Kaplan-Meier survival curve (B) for the whole study (n = 12). (C) The rechallenge experiment using mice surviving after CpG-STAT3dODN treatment as in p. A-B or naïve control mice. The CpG-STAT3dODN treatment resulted in long-term protection from tumor rechallenge.

757. AAV Gene Therapy Correction of Systemic Leak and Stress-Induced Edema in a Heterozygote Mouse Model of Hereditary Angioedema

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Hereditary angioedema (HAE), affecting 1 in 50,000 individuals from all ethnic groups worldwide, is a life-threatening autosomal dominant deficient disorder characterized by unpredictable, episodic attacks of swelling of the face, extremities, genitalia, gastrointestinal tract and upper airways. More than 99% of HAE cases are caused by mutations in the SERPING1 gene resulting in a deficiency in functional plasma C1 esterase inhibitor (C1EI), a serine protease inhibitor that normally inhibits proteases in the contact, complement, and fibrinolytic systems. If attacks are not treated in a timely manner, laryngeal edema can result in death by asphyxiation. Current treatment of HAE includes prophylaxis with attenuated androgens and management of attacks with plasma purified or recombinant C1EI, bradykinin and kallikrein inhibitors, all of which are complicated by cost, limited compliance, adverse effects and contraindications. As an approach to effectively treat HAE with a single treatment, we hypothesized that a one-time administration of an adeno-associated virus (AAV) gene transfer vector expressing the coding sequence of the normal human C1EI would provide sustained C1EI levels in plasma, sufficient to prevent angioedema episodes. To assess this hypothesis, we used CRISPR/Cas9 technology to create a novel C1EI deficient mouse model. The heterozygous mouse (S63 +/-) shares characteristics associated with HAE in humans including decreased plasma C1EI and C4 levels, increased bradykinin levels, and increased vascular permeability in the skin and internal organs. Administration of a serotype rh.10 AAV vector coding for human C1EI (AAVrh.10hC1EI) to the S63+/- mice resulted in sustained human C1E levels above the predicted therapeutic levels and complete correction of the vascular leak in the skin, paws and internal organs for at least 24 wk, the last time point evaluated. To demonstrate that the increased vascular permeability in the S63+/- mice was a direct result of C1EI deficiency, Evans blue dye was injected intravenously and extravasation of dye quantified. Compared to wild type mice under baseline conditions, untreated C1EI+/- mice had increased extravasation of the dye into the hind paws and multiple organs quantitated by optical absorbance at 600 nm. Strikingly, AAVrh10.hC1EI-treated (10^{11} gc) mice displayed a marked decrease in dye extravasation. To induce HAE attacks in S63+/- mice, carrageenan, a polysaccharide that induces inflammatory responses, was injected intradermally into the paws of untreated and AAVrh10.hC1EI-treated mice. Carrageenan induced progressive subcutaneous edema in untreated mice was significantly reduced in AAVrh10.hC1EI-treated mice as quantified by change in paw thickness over time. In summary, a single treatment with AAVrh.10hC1EI has the potential to provide long-term protection from angioedema attacks in affected individuals, resulting in long-term protection against the clinical phenotype.

758. Hematopoietic Stem Cell Gene Therapy for *Ifngr1* Deficiency Protects Mice from Mycobacterial Infections

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Mendelian Susceptibility to Mycobacterial Disease (MSMD) is a rare primary immunodeficiency, characterized by recurrent severe infections caused by otherwise weakly virulent mycobacteria. MSMD is associated with mutations in different genes, all leading to an impaired activation of macrophages (MΦ) by T-cells and a defective innate immune response. Specifically, mutations in the interferon-gamma-(IFNγ)-receptor-1 (*IFNγR1*) gene result in a life-threatening disease phenotype with most patients dying in early childhood. Here, we introduce the first successful hematopoietic stem cell (HSC) gene therapy approach for IFNγR1 deficiency using SIN lentiviral vectors, expressing Ifnγr1 either constitutively from an SFFV promoter (Lv.SFFV.Ifnγr1.iGFP) or cell type specific from a synthetic myeloid-promoter (Lv.SP146gp91phox.Ifnγr1.iGFP). Transduction of *Ifnγr1*$^{-/-}$ HSCs with each of the constructs led to stable *Ifnγr1* expression, which was sustained after differentiation towards MΦ and furthermore, restored the MΦ-related MSMD phenotype as shown by the correction of IFNγ-clearance, phosphorylation of STAT1 and the activation of downstream targets *iNos* and *Irf1*. In addition, genetic correction led to a normal T-cell activation pattern by restored up-regulation of HLA-DR and CD86 (B7.2) and was associated with correction of anti-mycobacterial activity against *Mycobacterium Avium* and *Bacille Calmette Guérin* (BCG) in HSC-derived MΦ. Moreover, transplantation of genetically corrected HSCs into *Ifnγr1*$^{-/-}$ mice revealed stable engraftment in the bone marrow as well as detection of corrected cells in spleen and lungs. Nine weeks after pulmonary infection by BCG-Pasteur (dsRed^{+}), severe signs of infection were observed in non-transplanted animals as represented by splenomegaly and pathological changes in lung and spleen, whereas *Ifnγr1*$^{-/-}$ mice receiving either WT or corrected HSCs showed only mild symptoms of mycobacterial infection. Improved disease parameters were in line with a decrease in mycobacterial burden in lung and spleen, which was further confirmed by light sheet microscopy demonstrating marked reduction of BCG infiltrates in the lungs of WT and corrected mice. Taken together, we here introduce the first HSC-based gene therapy

approach for MSMD using either constitutive or cell type specific expression systems, allowing to protect mice from severe mycobacterial infections and thus paving the way for a new therapeutic intervention.

759. Intravenously Administered FVIII Encoding Lenti-Viral Vectors Mediate up to 5000% of Normal and Persisting FVIII Expression in HemA Neonatal Mice

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Ex vivo gene therapy with lentiviral vectors (LV) for gene replacement has demonstrated clinical efficacy for multiple indications and with multi-year follow up in treated patients showing no evidence of tumorigenesis; Systemic delivery of LV-FIX mediates persistent FIX expression and is well tolerated in hemophilia animal models. The large packaging capacity, ability to sustain long-term transgene expression via gene integration, lack of pre-existing anti-LV antibodies (abs) in human populations and the encouraging in vivo profiles demonstrated in pre-clinical and clinical settings, make LV a promising vehicle for *in vivo* gene delivery, especially for gene candidates with large cDNA size such as Factor VIII (FVIII). To evaluate the potential use of LV-FVIII for the treatment of hemophilia A (HemA), codon optimized Human FVIII (hFVIII) variants placed under a hepatocyte-specific promoter were built into a LV system that contains multiple copies of microRNA-142 target sequences to minimize FVIII expression in antigen presenting cells and reduce the probability of inducing anti-FVIII abs. LV-hFVIII vectors were produced by transient-transfection of 293T cells, followed by 1000-fold concentration by ultra-centrifugation and evaluated in HemA mouse models. Post intravenous administration of LV-hFVIII, circulating hFVIII level was monitored by FVIII activity and antigen assays, LV transduction efficiency in the liver was assessed by measuring LV DNA copies via quantitative PCR and transgene RNA via In Situ Hybridization, anti-hFVIII abs were measured by total anti-hFVIII antibody ELISA. Persistent FVIII expression was observed for all LV-hFVIII variants in HemA mice that were treated at the neonatal stage; At 1.5E10 transducing units/kg dose, LV encoding codon optimized hFVIII (LV-cohFVIII) resulted in 30 to 100-fold higher circulating FVIII than LV encoding wild type hFVIII, while the vector copy number in liver cells and percent of FVIII RNA positive cells were comparable in all tested groups. Combination of codon optimization with XTEN (LV-cohFVIII-XTEN), a non-structured hydrophilic poly-peptide that presumably improves the circulating half-life by increasing the hydrodynamic size of the payload, resulted in 30-50 IU/mL FVIII activity in plasma, representing 3,000 to 5,000% of normal circulating FVIII level. Furthermore, anti-hFVIII abs were only detected in mice with supra physiological level of hFVIII, but no cytotoxic T lymphocyte response against LV transduced cells was observed in anti-hFVIII ab positive mice. Our result supports further development of LV-FVIII for in vivo gene therapy of hemophilia A.

Preclinical Progress Towards Therapies for Neurosensory Disorders

760. Application of CRISPR Technology as Treatment for Genetic Hearing Loss

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Mutations in a large number of genes are associated with genetic hearing loss in human for which there is no treatment. AAV-mediated gene therapy, anti-sense oligos and RNAi have been used to treat genetic hearing loss in animal models. For each technology clear limitations exist that need to be overcome for them to be clinically relevant. Rapid progress in CRISPR/Cas9-mediated genome editing technology has opened a new avenue to develop novel treatment for genetic hearing loss. Genome editing has the potential to permanently correcting mutations, by disruption or repair of mutations, with the lasting effect on hearing recovery. To use genome editing to treat genetic hearing loss it is essential to establish the approach is suitable for inner ear delivery that can minimize the risk such as off-target effect with demonstrated efficacy. We aim to achieve the goal by developing direct delivery of protein and nucleic acid complex into the mammalian inner ear. We will present the data to show application of CRISPR/Cas9-mediated genome editing to target a dominant mutation in Tmc1 gene in a mouse model with progressive hearing loss, Beethoven (*Bth*). We designed a series gRNAs to target the *Bth* mutation and performed in vitro assay to select the most specific gRNAs for in vivo study. Cas9 protein and gRNAs complexed with lipid formulation were injected into neonatal *Bth* mouse inner ear. Subsequent study showed significant hearing recovery across most frequencies. By high-throughput sequencing we demonstrated genome editing effects by the presence of indels at the mutation site in the injected inner ear. We further showed the hearing rescue effect is gRNA specific. Genome editing resulted in survival of auditory hair cells and improved startle reflex. Finally we detected very low off-target rate, a strong indication that protein-gRNA mediated genome editing could be ideal to treat genetic hearing loss. Our work demonstrates the combination of direct protein/RNA delivery with CRISPR/Cas9 mediated genome editing can target dominant mutations of hair cell genes, establishing a new treatment paradigm for dominant genetic hearing loss.

761. Gene Therapy Restores Auditory and Vestibular Function in a Mouse Model for Usher Syndrome Type 1C

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While there are no biological treatments currently available for inherited deafness, adeno-associated virus (AAV) based gene therapy is a promising therapeutic approach. Prior studies have been limited in their ability to target both classes of sensory auditory hair cells: inner hair cells that are the primary conduits for sound information transmitted to the brain, and outer hair cells that amplify and tune auditory input. We investigated the use of a synthetic AAV based vector, Anc80L65, to transduce both types of auditory hair cells as well as vestibular hair cells, in order to restore auditory and vestibular functions in an animal model for human Usher Syndrome, type 1C. The knock-in mouse model, *Ush1c* c.216G>A, carries a cryptic splice site mutation found in French-Acadian USH1C patients. The mutation results in expression of a truncated protein at the expense of the full-length protein, known as harmonin. Homozygous *Ush1c* c.216G>A mice (c.216AA) suffer from severe hearing loss, auditory hair cell degeneration, and vestibular dysfunction at one month of age. To explore the feasibility of gene augmentation therapy to treat USH1C, we characterized hair cell survival and function in postnatal c.216AA mice. We demonstrate that during the first postnatal week most c.216AA hair cells survive and retain hair bundles, albeit with reduced mechanotransduction current amplitude. Utilizing vectors expressing two splice forms of the wild-type *Ush1c* gene, we tested their therapeutic efficacy after round window injection into the cochlea of neonatal c.216AA mice. We demonstrate recovery of *Ush1c* gene and protein expression, successful targeting of harmonin proteins to the hair bundle or synapse, and restoration of hair cell mechanotransduction. This cellular level repair promoted increased hair cell survival, rescued complex auditory function, and recovered hearing and balance behavior to near wild-type levels. The data represent unprecedented recovery of inner ear function and suggest that biological therapies to treat deafness may be suitable for translation to humans with certain types of genetic inner ear disorders.

762. Allele-Specific Gene Editing for the Treatment of Autosomal Dominant Retinitis Pigmentosa

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PURPOSE: Targeted gene knockout is an emerging approach for the treatment of autosomal-dominant disorders such as P23H *RHO* mediated autosomal dominant retinitis pigmentosa (adRP), a common form of inherited blindness. A single nucleotide distinguishes P23H *RHO* from the WT copy which collectively present in a heterozygous manner. Previous works have suggested that down-regulation of the mutant allele in presence of the WT copy prevents retinal degeneration. Therefore, a 930nt *I-CreI* based designer homing endonuclease (HE) to specifically knockout the mutant RHO allele was engineered and validated in a self-complementary AAV vector. The specificity of this HE for P23H RHO was demonstrated *in vitro*. Preclinical evaluations in a human P23H RHO adRP mouse model were undertaken to determine if this approach demonstrates clinical potential for preventing vision loss in adRP patients.

METHOD: A proprietary ARCUS platform was employed to generate a HE that can specifically target the human *RHO* allele carrying the P23H mutation. The targeted 22 nucleotide sequence differs from the wild type allele only by a single base pair. Specificity of the nuclease was first evaluated in CHO cell lines engineered to contain a single strand annealing GFP reporter that harbors either the WT or P23H *RHO* sequence. Gene editing efficiency was evaluated by flow cytometry and immunoblotting. To evaluate HE activity *in vivo*, a scAAV5 vector harboring P23H RHO HE cDNA under control of a photoreceptor specific promoter was administered to the human P23H *RHO* adRP mouse model via sub-retinal injection. scAAV5-GRK-HE cleavage efficiency of the mutant *RHO* allele was calculated using sequencing and by digital PCR. *In vivo* retinal function was evaluated by electroretinography and the subcellular localization of rhodopsin in photoreceptors by histology.

RESULTS & DISCUSSION: We have generated a HE that is highly specific for the mutant P23H RHO allele. Sub-retinal delivery of scAAV5-GRK-HE decreases the amount of rhodopsin in outer nuclear layer in transduced photoreceptors when compared to non-transduced cells. This is the first study to demonstrate that a single chain HE can efficiently discriminate a one nucleotide difference and specifically target the P23H *RHO* allele. This result coupled with decreased rhodopsin mislocalization in an adRP model, foreshadows possible use of scAAV HE-based gene editing of P23H *RHO* to treat adRP patients.

763. A Potential Therapeutic Strategy for the Treatment of Autosomal Dominant Retinitis Pigmentosa

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Autosomal Dominant Retinitis Pigmentosa (adRP) is a rare genetic disease characterized by loss of photoreceptors resulting in a progressive loss of vision. Over 150 different mutations in the rhodopsin gene have been described, most leading to adRP. Mutant opsin proteins are prone to misfolding, aggregation, and incorrect intracellular trafficking. Dominance in RHO mutations can be associated with interference with the function of normal rhodopsin or intrinsic toxicity of the mutant protein. For interference, increased production of the wild type protein may be therapeutic, but for toxicity, suppression of the mutant protein could be necessary. We have developed a dual component AAV vector that incorporates both suppression and replacement elements targeted at human rhodopsin. The AAV vector harbors a miR sequence that targets both mutant and wild type rhodopsin alleles, in addition to a replacement human rhodopsin gene refractory to knock down by the encoded miR. This suppression/replacement vector could potentially be used to treat all forms of RHO associated adRP, regardless of mutation. A suppression replacement vector was designed that harbors a naturally occurring miR, which targets rhodopsin mRNA for degradation and/or translational repression through its target sequence, located in the 3' UTR of the rhodopsin mRNA. Additionally, the vector encodes a human rhodopsin gene refractory to the miR knockdown. Both elements are under the control of a rod, photoreceptor specific promoter. *In vitro* analysis of the suppression/replacement vector confirmed knockdown of both wild type and mutant rhodopsin, while knockdown was dependent on the presence of the miR 3'UTR target sequence. Additionally, the co-expressed replacement RHO was refractory to knockdown by the encoded miR. Moreover, subretinal delivery of an AAV5 suppression / replacement vector in the P23H RHO^{+} / $mRho^{+/+}$ transgenic model resulted in knockdown of both mutant P23H RHO and endogenous mouse Rho, with expression of replacement human RHO. A measurement of the integrity of the treated retinas by Optical Coherence Tomography (OCT) showed a significant improvement in the kinetics of retinal degeneration compared to retinas treated with control vector. In conclusion, these results highlight the potential of gene therapy in the treatment of adRP through the use of AAV delivery of a rhodopsin specific suppression/replacement vector targeting the photoreceptors of adRP patients.

764. Efficient *In Vivo* Gene Editing of Inherited Retinal Disease Genes in Mice and Non-Human Primates

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Recent successes in human clinical trials have demonstrated the safety and efficacy of subretinally injected adeno-associated virus (AAV) and established this as a promising approach for CRISPR-based gene editing therapies to treat a wide range of inherited retinal diseases (IRDs). While traditional gene augmentation approaches hold great promise for many ocular disorders, gene editing is uniquely positioned to address autosomal dominant diseases as well as those caused by mutations in genes whose size prohibits packaging in AAV or where overexpression has been shown to be toxic. Here we demonstrate the application of CRISPR/Cas9 for highly efficient targeted gene editing in the retina *in vivo*.

Leber congenital amaurosis (LCA) is the leading inherited cause of blindness among children. LCA comprises a genetically heterogeneous group of retinal disorders, with disease-causing mutations identified in over 20 different genes. One of the most common LCA-associated genes is *CEP290*, which exceeds the packaging limit of AAV and is therefore not amenable to a cDNA complementation approach. The most common mutation in *CEP290* is the IVS26 c.2991+1655 A>G mutation. This point mutation in intron 26 generates a novel splice donor, resulting in aberrant splicing and the presence of a premature stop codon. Using the *S. aureus* Cas9, we employed a dual-cutting approach in which two gRNAs are used to excise the mutation-containing region. In primary patient fibroblasts, we show that targeted deletion results in increased expression of wildtype *CEP290* and concomitant decrease in expression of the aberrantly spliced mutant RNA transcript. Using targeted amplicon sequencing of computationally predicted sites, as well as the minimally-biased GUIDE-Seq method, we selected highly specific guide RNAs (gRNAs) with no detectable off-target cleavage in a variety of cell types.

Critical to the success of a gene editing therapeutic for IRDs is the ability to efficiently deliver the Cas9 machinery and achieve sufficient levels of editing in mature photoreceptors. To this end, we have optimized the expression of our gene editing system in AAV5, a serotype previously shown to have strong tropism for rod and cone photoreceptors. To evaluate the *in vivo* efficacy of our approach, we constructed surrogate gRNAs targeting *CEP290*, as well as *GUCY2D*, the gene mutated in LCA1 and CORD6, in the mouse and cynomolgous macaque genomes. We delivered gRNAs and Cas9 via subretinal injection of AAV5 and show efficient targeted gene editing in both species. We utilized a novel sequencing method to accurately quantify not only indels, but

also larger deletions and inversions, which have typically been difficult to measure. Additionally, these experiments allowed us to compare various promoters, including a tissue-specific promoter that drives expression exclusively in photoreceptors.

Our experiments in patient cells establish proof of concept for the ability of gene editing to correct an LCA disease-causing splice mutation. Importantly, our *in vivo* experiments demonstrate targeted gene editing in the primate retina, a foveated species with ocular characteristics most similar to human. This first demonstration of *in vivo* gene editing in a primate retina supports the prospect of ocular gene editing and represents an exciting step forward in the development of CRISPR-based medicines to treat inherited retinal disorders.

765. A C-Terminal Fragment of CEP290 Is Sufficient for Rescuing Vision in Mouse Model for LCA Due to *Cep290* Mutation

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Leber Congenital Amaurosis (LCA) is an inherited retinal degenerative disorder affecting 1 in every 80,000 live births. Mutations in *CEP290* gene account for approximately 22% of total LCA cases. Gene replacement therapy for *CEP290*-LCA was hindered by the large size of the wild-type (WT) CEP290 coding sequence that exceeds the packaging limit of an adeno-associated viral (AAV) vector. To circumvent this problem, we tested whether a part of the CEP290 coding sequence can provide therapeutic effects in a mouse model of the disease. Four different truncated versions of the CEP290 coding sequence were packaged into AAV8 capsids and were tested in an *rd16/Nrl-KO* double mutant mouse line which models cone photoreceptor degeneration of *CEP290*-LCA patients. These mice exhibit compromised cilia structure with diminished cilia proteins in the photoreceptors and completely lose their cone function in approximately four months after birth. Electroretinography (ERG) and optomotor test were performed to monitor the retinal function and visual behavior of these mice following vector delivery. Among the vectors tested, a C-terminal CEP290 protein fragment was found to be very effective in preserving the retinal function in *rd16/Nrl-KO* mice over an eight-month period. The mice also exhibited a significantly improved optomotor response in the vector-treated eyes than the control eyes. The C-terminal CEP290 protein fragment was localized to the connecting cilia of the photoreceptors following vector delivery, and the treatment was able to maintain the normal expression and localization of a few CEP290-interacting cilia proteins, as revealed by immunofluorescence analysis. The treatment also preserved a relatively normal structure of the connecting cilia as shown by electronic microscopy. Trafficking of photo-transduction proteins including S-opsin, M-opsin and cone PDE appeared normal as well. CRISPR/Cas9 mediated *in vivo* knockdown of the mutant CEP290 together with co-immunoprecipitation studies revealed interdependency of the C-terminal CEP290 fragment and the mutant form of CEP290 in the mice for functional rescue. Our study established that an LCA-causing *CEP290* mutation can be complemented *in-trans* by a truncated version of the WT protein, which provides a new insight into the therapy development for diseases associated with *CEP290* mutations.

766. Gene Supplementation Therapy for *CNGA3*-Linked Achromatopsia

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Achromatopsia (ACHM) is a genetically and clinically well-defined inherited retinal disorder. Patients with ACHM suffer from severely impaired daylight vision, characterized by poor visual acuity, photophobia, nystagmus (involuntary rapid eye movements), and lack of the ability to discriminate colors. Currently, six disease genes have been identified including the two genes encoding the cone-specific cyclic nucleotide-gated channel subunits *CNGA3* and *CNGB3*. We developed a recombinant adeno-associated virus (AAV) vector-mediated gene supplementation therapy for the treatment of CNGA3-linked ACHM (ACHM2). The vector expresses full length human *CNGA3* under control of the human, cone-specific cone arrestin promoter and was packaged with AAV8 capsid. The resulting vector (rAAV8.CNGA3) was tested for efficacy in the *Cnga3* knockout (KO) mouse model of ACHM2. Toxicity and biodistribution was assessed in non-human primates (NHP). rAAV8.CNGA3 delivered into the subretinal space of *Cnga3* KO led to efficient and stable CNGA3 transgene expression and biological activity as determined by immunohistochemistry and electroretinography, respectively. Up to $1x10^{12}$ total vector genomes (vg) have proven save when delivered into the subretinal space of NHPs with only limited to modest biodistribution and shedding and minimal signs of inflammation. An interventional phase I/II clinical trial (NCT02610582) was initiated focusing on safety and efficacy of a single subretinal injection of rAAV8.CNGA3 in patients with ACHM2 at three different doses: $1x10^{10}$, $5x10^{10}$, and $1x10^{11}$ total vg. The approach was targeting the central retina and involved temporal detachment of the fovea / macula. So far, the treatment has proven to be safe, was well tolerated and did not result in any clinically apparent inflammation or test item related events. Preliminary clinical data will be discussed.

767. Directed Evolution of Intravitreally Delivered Adeno-Associated Viral Vectors in the Non-Human Primate Retina

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Introduction: Inherited retinal degenerations are caused by mutations in over 200 genes, and the majority of these mutations phenotypically converge with the gradual loss of photoreceptors, making this cell type the primary therapeutic target. Gene therapy is a promising treatment strategy for these patients, but current adeno-associated virus (AAV) vectors are limited in their ability to transduce photoreceptors following the most promising surgical route of administration, an injection in the vitreous fluid of the eye. In addition, due to substantial differences in the size and structure of primate vs. rodent eyes, including the presence of a macula, species-specific differences in expression has been observed in AAV-mediated delivery in the eye and argues that vector screens should be performed *in vivo* in the non-human primate (NHP). In this study, highly diverse AAV variant libraries were intravitreally injected in NHPs and selected for photoreceptor delivery.

Methods: Multiple peptide-insertion libraries were generated based on naturally occurring and engineered AAV serotypes, which have strong tropism for RPE and photoreceptors but lack the ability to infect the outer retina from the vitreous. The libraries were then intravitreally injected into NHP eyes. Samples of retinal tissue were collected from different retinal regions, and variants were then recovered and repackaged for additional rounds of selection. Next generation sequencing was used to characterize the convergence of AAV libraries over all rounds of selection and to identify variants capable of delivering DNA cargo to the outer retina, particularly photoreceptors. Lead candidates were identified from each of the libraries, and the tropisms of variants were characterized through immunohistochemistry and deep sequencing.

Results: Variants were successfully recovered from each round of selection from each area of the retina tested. Deep sequencing analysis was able to identify variants that had the highest fold change in representation from the initial library to the final round of selection. Lead candidate variants were identified from each of the libraries and packaged with a GFP reporter fused to a corresponding barcode. Deep sequencing analysis and immunohistochemistry of barcoded library members resulted in the identification of multiple candidate variants with different expression properties that showed improvement over existing vectors.

Conclusions: This study demonstrated that *in vivo* directed evolution of AAV in the primate retina resulted in variants with significantly improved tropism for the outer retina compared to existing serotypes. The variants show promise for the treatment of a wide variety of retinal dystrophies, and may allow for safer and more efficient panretinal gene therapy in patients. Ongoing studies are being performed to further characterize the potential of identified vectors for clinical applications.

Engineering and Manufacturing Vectors and Cells II

768. Donor-Derived Sleeping Beauty Engineered CAR CD19 Cells for B-Acute Leukemia

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CD19-specific CART cells demonstrated promising positive results, achieving durable responses in relapsed and refractory patients affected by B-lineage leukemias. We recently established a platform for non-viral gene manipulation of Cytokine-Induced Killer (CIK) cells, an effector T cell population characterized by enrichment in CD3+CD56+ cells and reduced risk of GvHD, in compliance with Good Manufacturing Practices (GMP).

In this study, we evaluated the feasibility and reproducibility of a GMP-compliant protocol, and the preclinical efficacy and safety of SB modified CARCIK-CD19 cells. Large scale manufacturing process was verified starting from 30-60X10^6 PBMC, reaching stable expression of CD19CAR (average 65%). Modified cells displayed a specific and effective cytotoxicity and proliferation towards CD19-positive cell lines and primary tumors. We manufactured three lots by seeding 46.0x10^6, 56.8x10^6 and 140.4x10^6 PBMCs respectively in an academic Cell Factory, authorized by Agenzia Italiana del Farmaco (AIFA). After 21-22 days of culture, we harvested 15.998x10^9, 1.436x10^9, and 3.897x10^9 total nucleated cells respectively, with a mean viability of 96.96%. The median expression of CD3+CD19CAR positive cells was 46.90% (range 31.27% - 65.45%). Frozen/thawed CD19.CAR CIK cells remained active *in vitro* and in an established MLL-ENL patient-derived xenograft model. Furthermore, CARCIK-CD19 cells showed a dose-dependent antitumor response and persisted in a xenograft mouse model of common BCP-ALL, bearing the feature of a Ph-like gene rearrangement (PAX5/AUTS2), and in a survival model with Daudi-cell lymphoma. CARCIK-CD19 cells induced complete eradication of disseminated tumor at 100 days while untreated mice had a median survival of 40 days. Finally, infusion of CARCIK-CD19 cells proved to be safe and well tolerated in a xenograft model of bio-distribution and toxicity. The infused cells persisted in time in the hematopoietic and post-injection perfused organs with a follow-up of 3 months.

Our findings describe a novel donor-derived non-viral CAR approach characterized by efficient cell transfection, expansion and functionality that could be used as valid and sustainable alternative to patient-derived viral approach. This study provides the proof-of-concept for designing phase I/II study for relapsing and refractory ALL post Hematopoietic Stem Cell Transplantation.

769. CD46 Receptor Knockout in Packaging Cell Lines Improves Production of Measles Glycoprotein Pseudotyped Lentiviral Vectors and Transduction Efficiency of Human CD34+ Cells

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Producing lentiviral vectors (LVs) with alternate viral envelopes, known as pseudotyping, significantly widens the range of targetable cell types for transduction by LVs. Vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped LVs are the standard for human hematopoietic stem and progenitor cells (HSPCs) gene delivery. However, a limitation of VSV-G pseudotyped LVs for clinical gene delivery applications is their inability to efficiently target long-term repopulating stem cells. Our group has proposed that this low efficiency is due to a block in pH-triggered vector escape from the endosome. We reasoned that vectors with alternate entry strategies may be able to avoid this block.

Previous studies by our group and others demonstrated that measles virus could infect HSPCs with CD46 as the predominant receptor, and that virus may enter at the cell surface in a process resembling macropinocytosis. LV vectors pseudotyped with the Edmonston vaccine strain measles virus (MV) hemagglutinin (H) and fusion (F) glycoproteins have shown enhanced ability to transduce resting lymphocytes and monocyte-derived dendritic cells compared to VSV-G LVs. Edmonston strain MV hemagglutinin recognizes the ubiquitously expressed CD46 protein for viral entry, unlike wild-type virus strains that utilize CD150 or Nectin-4. Herein, we show that MV LVs have up to 3-fold enhanced capability to transduce cytokine stimulated cord blood (CB) derived HSPCs as compared to VSV-G LVs at an equivalent multiplicity of infection (MOI) (mean %EGFP VSV-G =10% vs. MV = 35% at MOI 1). This improved efficiency may arise from the use of an alternate, pH-independent entry mechanism, as VSV-G enhancing small molecules such as rapamycin, PGE2 and β-deliverin did not improve MV LV transduction.

Despite this improved transduction efficiency, low titers during vector production by transient transfection limit MV LV applicability. We show that CD46 expression on H/F transfected HEK 293T packaging cells is sufficient to induce adjacent cell membrane fusion, resulting in multinucleate syncytia and cell death, prior to peak vector production in cell supernatant. We reasoned that removal of CD46 on the packaging line could limit cell death, thus permitting an LV preparation that would be free of cell debris, and perhaps result in higher titer.

Consequently, we pursued the CRISPR/Cas9 mediated knockout of CD46 in HEK 293T cells (CD46 KO), followed by cell selection and flow cytometry sorting. MV LVs produced in CD46 KO cells produced 2-fold higher titer vector, with approximately 2-fold higher p24 protein concentration, compared to MV LVs produced in wild type HEK 293Ts. CD46 KO HEK 293Ts also demonstrated no syncytia formation and continued cell viability throughout vector production.

When matched for MOI, MV LVs generated in CD46 KO cells resulted in approximately 2-fold higher transduction in CB derived HSPCs, compared to vector produced in wild type HEK 293Ts (mean %EGFP for WT MV LV =29% vs. KO MV LV = 62% at MOI 1). This enhanced transduction efficiency was observed despite less p24 protein per transducing unit in virus generated in KO versus wild type cells.

Given that vector production and quality are major sources of cost and variability in clinical trials of gene therapy, we reason that the use of CD46 KO packaging cells may lower the cost and improve the efficiency of generating MV LVs. The large-scale use of these cells is compatible with current methods for improving the purity and increase the yields of LV produced. Further mechanistic investigations comparing the improved transduction efficiency of MV LVs compared to VSV-G LVs are ongoing, with the goal of identifying restrictions in HSPC transduction that may prevent efficient gene delivery generally.

770. Advancing rAAV QC Pipelines with AAV-GPseq: Abundant Detection of Chimeric Genomes in Preparations and Its Implications for Vector Biology

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Studies regarding the efficacy and safety of recombinant adeno-associated viruses (rAAVs) often overlook whether packaged vector genomes are full-length molecules and whether they may harbor non-vector genome sequences. It has long been shown that fragmented or truncated wild-type AAV genomes can be packaged to form defective interfering particles. However, the extent and frequency of occurrence during rAAV production is unknown. Using single-molecule, real-time (SMRT) sequencing and custom bioinformatic pipelines, we have recently shown that truncated genomes can severely dominate rAAV-shRNA preparations, compromising the purity and potency of rAAVs produced. We have now expanded upon our methodology, named AAV-genome population sequencing (AAV-GPseq), to examine the composition of entire populations of self-complementary AAV genomes in vector preparations. By employing phage DNA spike-ins and normalization steps to overcome SMRT-sequencing loading bias, we can accurately assess the relative distribution of truncated genomes versus full-length genomes in preparations. Importantly, AAV-GPs allows for single-particle sequence resolution from ITR-to-ITR. For the first time, AAV-GPseq can ascertain the distribution of flip and flop orientations at both ITR ends from a continuous unbroken sequence.

With AAV-GPseq, we were able to detect many reverse packaged genomes that encompass bacterial sequences originating from the cis-plasmid, as well as detect genomes with short adenoviral helper, rep, or cap gene sequences recombined with ITR sequences. We discovered that highly complex recombination events occur to yield chimeric rAAV genomes containing sequences originating from multiple sources, packaged as a single molecule. These particles are detected with frequencies as high as 5% of vector preparations. Most intriguingly, we detected an abundance of packaged host cellular DNA sequences

recombined with AAV ITR elements. This suggests that packaging of non-specific DNA may not be passive events, but rely on recognition of potential Rep-binding sites on chimeric molecules. Most puzzling is the observation that many chimeric sequences mapped to gene promoter sequences. This discovery might beg the reevaluation of the extensive reporting of rAAV integration into gene promoter regions. Alternatively, some of these occurrences may be attributed to chimeric sequences that have been packaged into rAAV to yield false-positive integration events.

Although chimeric sequences are on average shorter than 0.5 kb in length, and therefore do not encompass full genes, these discoveries still pose new concerns for how we define the purity of viral vector preparations. Our work introduces unique next generation QC pipelines to the field of gene therapy and in many respects responds to a world of rapid clinical development and commercialization for rAAV gene therapy.

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771. Development and Optimization of a Serotype-Independent Method of Adeno-Associated Virus Harvest and Purification

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Introduction: AAV is the vector of choice for over 7% of all gene therapy clinical trials, with 173 clinical trials open worldwide as of August 2016. However, production of clinical material often relies on inefficient (e.g., size exclusion chromatography, cesium chloride centrifugation) or expensive and highly serotype-specific (e.g., affinity) downstream processes. Therefore, we investigated a scalable and serotype-independent alternative primary capture and purification method. Here, we evaluate a hydrophobic interaction chromatography membrane for its ability to capture and purify AAV1, AAV3-like, AAV5, and AAV8 particles from cell culture media and cell lysates. We not only demonstrate efficient capture and purification but also demonstrate a technique that allows integration of cell lysis and downstream processing. **Methods**: AAV particles were produced in adherent HEK 293T/17 cells using either serum-free DMEM or DMEM supplemented with 1-5% FBS. Cultures were maintained between 3 and 6 days post-transfection, at which time culture media was decanted and cells detached and lysed by freeze/thaw or using concentrated ammonium sulfate or NaCl solutions. AAV-containing fractions were titrated to the appropriate salt concentration and applied to a Sartobind Phenyl hydrophobic interaction chromatography membrane. The optimum conditions for recovering AAV8 from the membrane were determined using design of experiment (DoE) analysis. Load, flow-through, wash, and elution fractions were analyzed by AAV capsid ELISA to determine purification efficiency for all serotypes. Binding capacities were determined for AAV8 and AAV3-like particles, and the reduction in host cell proteins and dsDNA over the phenyl membrane were assessed. **Results**: DoE results showed that the combinations of 1.5 M ammonium sulfate with pH 7.5 and 1.25 M ammonium sulfate with pH 7 provided comparable recoveries, with up to 100% recovery of the loaded AAV particles. However, 1 M ammonium sulfate solutions recovered less than 40% of loaded AAV8, regardless of pH. It was also established that lysis of AAV-containing cell pellets using high concentrations of ammonium sulfate or NaCl provided equivalent efficiency to lysis using freeze thaw, and direct application of these lysates to the HIC membrane showed that binding and recovery was substantially higher using ammonium sulfate. This lysis technique also allows for substantial removal of dsDNA-containing insoluble impurities prior to membrane chromatography. AAV recovery from the phenyl membrane was over 87% with lysates and media from AAV1, AAV3-like, and AAV8 particles, and over 76% with AAV5 fractions. The dynamic binding capacity of the phenyl membrane was determined to be 2.3×10^{13} capsids/mL of membrane at 10% breakthrough for AAV8, and 1×10^{13} capsids/mL of membrane at 10% breakthrough for AAV3-like particles. The phenyl membrane provided a greater than 90% reduction in total host cell protein and a greater than 80% reduction in dsDNA from the load to the elution fraction. **Conclusions**: The phenyl ligand provides highly effective purification of multiple serotypes of AAV from both media and lysates. The use of a high salt buffer provides for effective capture and release of AAV, with a substantial reduction of dsDNA-containing insoluble impurites. This has important implications for large scale production as this technique is amenable to either suspension or fixed bed adherent cell culture. This simple, robust, and scalable process integrates release, capture, and purification of AAV vectors and is compatible with multiple industrially relevant serotypes of AAV.

772. RNA Interference Mediated Ablation of Cytotoxic Transgenes Enables Productive Viral Vector Packaging

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Viral vector mediated gene transfer is a valuable tool for studying gene functions and gene therapeutics. But some transgene products are toxic to the packaging cells or incompatible with the package system, leading to either very low titer or no production of viral vectors. We hypothesize that in such cases, abolishing the transgene expression by RNA interference (either shRNAs or artificial miRNAs, amiRNAs) during the vector packaging process would enable efficient vector production. In our proof-of-concept studies, we tested our hypothesis in production of two lentiviral vectors carrying EGFP-$(GR)_{80}$ (Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) related dipeptide repeat protein) or ApoL1 (apolipoprotein L1) gene. These two vectors cannot be packaged by using conventional production procedure. However, co-transfection of plasmid expressing shRNA specific to the EGFP-$(GR)_{80}$ fusion transcripts successfully generated high titer and functional Lenti-EGFP-$(GR)_{80}$ vector. Moreover, we reasoned that more efficient silencing of transgenes during the vector packaging and a more generic strategy that can be versatilely applied to the same vector genome backbone carrying different transgenes may further improve the simplicity and efficiency of viral vector production. Therefore, we incorporated three copies of target sites for either shRNAs or artificial miRNAs in the 3'UTR of ApoL1 transgene in

the Lenti-ApoL1 plasmid. Those target sites were designed not to be recognized by any known mammalian endogenous small RNAs but specifically sensitive to the shRNA or amiRNA expressed from a co-transfected plasmid in the packaging process. We demonstrated that expression of transgene embedded with the artificial small RNA target sites is not compromised but is efficiently silenced in the presence of the corresponding shRNA or amiRNA. By doing so, the Lenti-ApoL1 vector was successfully produced with a high titer. Infection of HEK293 cells with Lenti-ApoL1 caused massive cell death, indicating the infectivity of packaged Lenti-ApoL1 vector and the toxicity of the ApoL1 gene. Here, we demonstrated that suppression of the cytotoxic or incompatible transgenes during vector production by transient RNA silencing enables the production of high titer and functional viral vectors that cannot be produced by conventional method. This strategy may be also applied for the package of other viral vectors (Ad, lentiviral vectors, etc.) carrying cytotoxic or incompatible transgenes.

773. Microfluidic-Enabled Manufacture of Nucleic Acid-Based Lipid Nanoparticles: Modulating Gene Expression from Bench Towards the Clinic

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Purpose: In recent years, demand for an efficient tool capable of delivering payloads for modulating gene expression *in vitro* and *in vivo* has been growing steadily. Lipid nanoparticles (LNPs) utilize endogenous delivery pathways that co-opt apolipoprotein E (apoE) to mediate effective delivery of the encapsulated nucleic acids into cells via the low-density lipoprotein receptor (LDLR). However, their transition from the bench to clinic has been considerably limited by challenges in manufacturing at both small and large scales. Here, we bridge that gap by describing the robust manufacture of potent lipid-based nanoparticles using an optimized microfluidic platform for highly efficient encapsulation of nucleic acids (e.g. siRNA, gRNA, mRNA, plasmid DNA) at scales suitable for discovery through to scale-up development.

Methods: Nucleic acids (siRNA, mRNA, plasmid DNA) were encapsulated into LNPs using the NanoAssemblr™ Platform (Precision NanoSystems Inc., Vancouver, Canada). LNPs at microliter scale were prepared using the NanoAssemblr Spark; for batches of < 10 mL, the NanoAssemblr Benchtop was used; and, LNP volumes > 10 mL were manufactured on the NanoAssemblr Blaze. The prepared LNPs were characterized for their size, PDI, and encapsulation efficiency. We further provide evidence of the efficient cellular uptake of these LNPs in primary and iPS-cells, including primary and iPS neurons. Knockdown of targeted mRNAs via siRNA mediated degradation, expression of exogenous mRNA sequences, and exogenous expression of genes cloned into plasmids, was determined.

Results: The nucleic acids were successfully encapsulated into LNPs using the NanoAssemblr™ platform. The LNPs exhibited similar sizes (<120 nm), low PDI (<0.2), and high encapsulation efficiency (>90%) across all three instruments. Flow cytometry and confocal microscopy analysis of 1 µg/mL GFP mRNA-LNP prepared on the NanoAssemblr Spark and Benchtop after 48 h treatment in rat primary neurons at DIV 7 showed similar (>95%) uptake of nanoparticles leading to >90% cells expressing GFP. Furthermore, 2.5 µg/mL GFP plasmid-LNPs exhibited >95% uptake of nanoparticles leading to >50% cells expressing GFP for both instruments. The high expression and uptake did not significantly impact cell viability as measured using Presto Blue Cell Viability Assay. Similarly, HPRT siRNA-LNPs prepared on the NanoAssemblr Spark and Benchtop showed similar (>80%) knockdown of HPRT gene in rat primary neurons after 48 h treatment. This provides for the seamless scale-up of optimized conditions from the discovery phase (100 - 1000 µL), to the benchtop instrument (1 mL - 15 mL), up to the pre-clinical stage (10 mL - 1000 mL).

Conclusions: The NanoAssemblr microfluidics-based platform supports pre-clinical development of nucleic acid-lipid nanoparticles by enabling seamless nanoparticle manufacturing from microliters to liters.

774. Optimizations in the Production of DNA Ministrings, Linear-Covalently Closed DNA Minivectors for Use in Non-Viral Gene Therapy

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Gene therapy requires successful transfer of a gene of interest past cellular and nucleic barriers. While non-viral gene therapy strategies aim to address the safety concerns inherent in viral vectors, they generally suffer from low transfection efficiency. The conventional plasmid vector contains many unnecessary and immunostimulatory elements for replication in prokaryotic systems, such as antibiotic resistance genes. We previously constructed an enhanced linear covalently closed (LCC) minivector, the DNA ministring, which confers improved efficiency and safety over its plasmid, and even its isogenic circular counterparts. The DNA minivector is comprised of the eukaryotic expression cassette without the bulk of an immunogenic bacterial backbone, thus ensuring greater bioavailability, higher transfection efficiency, and prolonged duration of gene expression. Furthermore, the LCC DNA ministring has covalently ends that protect against immediate degradation within the cell while the linear nature of the minivector minimizes the potential for insertional mutagenesis from random genomic integration.

DNA ministrings are produced through a simple heat-inducible bacteriophage (phage) -derived recombination system in recombinant *Escherichia coli*. The phage-derived protelomerase, Tel, regulated via a temperature-inducible expression system, acts on a parent plasmid to produce DNA ministrings *in vivo*. We present optimizations to our DNA ministring production process. We demonstrate improvements to the rate of parent plasmid processing into ministrings by encoding a second protelomerase into our platform. By encoding a homing endonuclease into the genome under control of a separate promoter, and its respective target site onto the backbone of the parent plasmid, we examine the potential for eliminating residual parent plasmid and LCC backbone and simplifying the purification of ministrings.

775. Persistent Genetic Modification of Dividing and Differentiating Cells with Non-Integrative and Autonomously Replicating DNA Vectors

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Introduction: The persistent genetic modification of cells with reporter genes is a useful technique, which allows them to be tracked and monitored in culture *in vitro* or in model systems *ex vivo*, and utilised in a variety of applications. A limitation of commonly used integrating vector systems is that, ideally, a vector should deliver sustained levels of transgene without compromising the vitality of the recipient host cell in any way. Here, we describe the use of Scaffold Matrix Associated Region (S/MAR) DNA vectors as a tool for genetically modifying differentiating and dividing cells. These novel cell lines are useful for developing cell models in culture and following ex vivo administration. The DNA vectors are autosomally replicating molecules which can provide robust and sustained transgene expression. In addition, their use does not preclude the subsequent application of other molecular or therapeutic protocols, such as drug treatments or tumour killing using oncolytic viruses. **Results**: Neuroblastoma cells derived from the bone marrow of a 2-year-old patient were stably modified with either GFP or Luciferase S/MAR DNA vectors, which provided robust and persistent expression of the transgene. We demonstrate that the DNA vectors replicate autosomally and remain episomal in these novel cell lines. We also show that the cell lines' behavior *in vitro* and *ex vivo* is unchanged following the genetic modification. We evaluated the ability of the S/MAR DNA vectors to sustain expression during a differentiation-like process with all the epigenetic changes that this involves. We forced the cells to differentiate into neurons by treatment with Retinoic Acid (RA) where they showed morphological changes including elongation and neurite extensions. Subsequently, the cells clustered into neurospheres and stopped proliferating. At that point, the cells still expressed robust levels of transgene. We also evaluated the DNA Vector's ability to be retained throughout the reverse process in which the neuron-like cells were allowed to regress into to their original neuroblastoma state by the withdrawal of RA. They recovered their cancer-like morphology and their capacity to proliferate. The molecular analysis of these S/MAR labeled cells reveals that the DNA vectors were retained at low copy numbers throughout the procedure and could be isolated and rescued from the cells' nuclei as episomes. In addition, immunocytological analysis of parental and DNA vector labeled cells show no difference in the expression of neuronal markers, suggesting that the vectors do not modify the cell's molecular profile. Finally, we engrafted genetically labeled cells by subcutaneous or intracranial injections into SCID mice. These cells formed representative tumours, which are comparable to the parental line with robust and undiminishing reporter gene expression. **Conclusions:** This data demonstrates that our S/MAR DNA vectors are capable of genetically modifying dividing and differentiating cells and can provide sustained robust expression of transgenes during "differentiation" and "de-differentiation" processes. We demonstrate that they are a valuable genetic tool that can be used to generate sophisticated isogenic cell lines without molecular or genetic damage.

Cancer Immunotherapies via Bacteria, Viruses and Novel Modalities

776. Development of an Effective Cancer Vaccine Platform Using Attenuated Salmonella typhi

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Poor antigen delivery is one of the major limitations of modern cancer vaccine vectors. To overcome this challenge, we exploited Salmonella Pathogenicity Island 2 (SPI2) and type III secretion system (T3SS) to deliver tumor-associated antigen (TAAs) into the cytosol of antigen-presenting cells (APC) in situ. In a recent report, we demonstrated that an attenuated strain of S. typhimurium, MvP728, which was engineered to express SPI2-regulated oncoprotein survivin (SVN), induced potent CD8 T-cell-mediated antitumor response that was curative in a murine model of highly aggressive lymphoma. In further development of this technology for clinical use, the vaccine has been transferred from an experimental strain of S. typhimurium to a clinically validated strain of S. typhi, CVD908. To adapt CVD908 to stably express recombinant antigens without antibiotic-dependent selection, we used a recently reported plasmid stabilization system that encodes the single-stranded binding protein (SSB), an essential protein in DNA metabolism, which was deleted from the bacterial chromosome. The SPI2-regulated expression cassette was then cloned into the SSB plasmid, so that the resultant construct maintained bacterial vector stability while expressing and translocating TAAs. We found that CVD908Δssb vector effectively infects human dendritic cells in vitro and translocates recombinant human SVN and MYCN oncoproteins into their cytosol. Furthermore, CVD908Δssb remains stable in mice and induces generation of antigen-specific CD8 T cells. Therapeutic vaccination of tumor-bearing mice with SVN or MYCN vaccine produced potent antitumor activity in murine models of lymphoma or neuroblastoma. The results justify clinical testing of CVD908Δssb-based SVN and MYCN vaccines in cancer patients. Supported by grants from CPRIT (RP121035) and The Leukemia and Lymphoma Society.

777. Non-Clinical Safety, Immunogenicity and Antitumor Efficacy of Live Attenuated Salmonella Typhimurium-Based Oral T-Cell Vaccines VXM01m, VXM04m and VXM06m

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VAXIMM is developing first-in-kind *Salmonella* typhi Ty21a-based oral T-cell vaccination platform for the initiation of anti-tumor cellular immune responses via a unique mode-of-action. This study summarizes the non-clinical safety profile, as well as the immunogenicity and anti-cancer efficacy of *Salmonella* typhimurium vaccines VXM01m, VXM04m and VXM06m, which encode murine vascular endothelial growth factor receptor 2 (VEGFR2), mesothelin (MSLN) and Wilm's tumor 1 (WT1) protein antigens respectively, in different mouse models.

The non-clinical safety profile of the control Salmonella *typhimurium* empty vector VXM0m_empty, as well as VXM01m, VXM06m and the VXM01m/VXM04m combination was assessed in C57BL/6J mice after repeated administrations by gavage with doses up to 10^8 CFU in 13- and 26-week GLP-compliant toxicology studies. Immunokinetic studies were performed in healthy C57BL/6J mice treated with doses up to 10^{10} CFU of either VXM0m_empty, VXM01m, VXM04m or VXM06m, and antigen-specific T cells were detected in the spleen by flow cytometry using MHC class I pentamers. The anti-tumor efficacy of VXM01m and VXM04m was evaluated in the Panc02 syngeneic mouse model of pancreatic adenocarcinoma overexpressing MSLN, and the anti-cancer activity of VXM06m was evaluated in the FBL-3 disseminated model of erythroleukemia expressing WT1.

VXM01m, VXM06m as well as the VXM01m/VXM04m combination were generally well tolerated in all the animal studies conducted. Treatment-related effects were limited to an increase in the number of animals with inflammation/single cell necrosis in the liver, which was attributed to the *Salmonella* vector. Moreover, treatment with each individual vaccine induced antigen-specific systemic immune responses peaking 7 to 10 days after the last dose. Treatment of mice with VXM01m and VXM04m single agents prior to the challenge with Panc02 tumor resulted in a significant reduction in the tumor growth rate compared to the control group, with a median T/C of 37.6% and 19.4% respectively, 35 days after tumor challenge. Treatment of mice with VXM06m prior to the induction of FBL-3 leukemia generated a rapid and sustained anti-tumor effect with 100% (10 out of 10) of surviving animals 175 days after leukemia challenge. In contrast, treatment with VXM0_empty did not show any anti-cancer effect, with a median survival of 45 days and 0% of cancer regression.

VXM01m, VXM04m and VMX06m were tolerated at the effective doses and demonstrated substantial immunogenicity and strong anti-cancer activities in different animal models. This study provides further evidence that VAXIMM's versatile oral T-cell vaccination platform can be used to stimulate anti-tumor immunity against various tumor-associated antigens. Further studies of VAXIMM's cancer vaccine candidates, as monotherapy and in combination with immune checkpoint blocking modalities, are warranted.

778. Targeted Interferon Gene Delivery Reprograms the Tumor Microenvironment and Induces Protective Immunity Against Multiple NeoAntigens

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Immunotherapy is emerging as a new pillar of cancer treatment with the potential to cure. The development of novel therapeutics targeting immune checkpoints has led to unprecedented rates of durable responses in tumors previously considered invariably lethal, such as metastatic melanoma. However, many patients fail to respond to these new therapies, either due to the failure to generate tumor-specific T cells or the existence of an immunosuppressive tumor microenvironment, which imparts resistance to blockade of CTLA4 or PD1/PDL1 checkpoints. To extend the benefits of current therapies, we express an Interferon-alpha (IFNα) transgene in the tumor infiltrating monocyte progeny of transplanted, genetically engineered hematopoietic stem cells and test whether our IFN cell and gene therapy could boost the induction consolidation and maintenance of anti-tumor responses and synergize with other immunotherapies in a novel mouse B-cell lymphoblastic leukemia (ALL) model mimicking human B-ALL. When we challenged the mice with our disseminated ALL model we found tumor growth inhibition in IFN mice. Administration of αCTLA4 blocking antibody had no effects in control mice but significantly improved ALL inhibition in IFN mice, suggesting an immune contribution to the observed response of IFN mice. To investigate the induction of anti-tumor immunity we generated a ALL variant expressing the ovalbumin model antigen (OVA-ALL) and showed effective OVA-ALL growth inhibition in IFN mice accompanied by induction of OVA-specific T-cells, with increased cytotoxic activity. Of note, a fraction of IFN mice generated durable responses and spreading of the immune repertoire to multiple tumor-associated antigens, which conferred long-term protection against both OVA+ and parental ALL. Depletion of CD8 T cells abrogated efficacy, confirming their major contribution to the anti-tumor response. In contrast, control mice generated fewer endogenous OVA-specific T cells which were hypo-functional and experienced high rates of immune-escape due to loss of OVA expression. Analysis of the tumor microenvironment revealed an ISG/Th1 gene signature and M1-skewed immune cell infiltrate in IFN mice. Conversely, the tumor injected controls showed increased content of MHCII-negative macrophages, consistent with the acquisition of an M2-like pro-tumoral phenotype, and expansion

of immature myeloid cells, which promote immune-suppression and tumor growth. Remarkably, adoptive transfer of OVA-specific T cells (OT-I) had limited efficacy in control mice, and showed lack of expansion and phenotypic evidence of exhaustion, similarly to the endogenous OVA-specific T cells. Conversely, when we combined our IFN gene and cell therapy with the adoptive transfer of OT-I cells or with αCTLA4 blocking antibodies, we significantly improved survival rates. These findings warrant the clinical testing of our strategy in patients undergoing disease remission after standard therapy, with the aim to provide long-term disease control. Hematological malignancies that foresee HSC transplantation as standard-of-care consolidation treatment might provide a suitable framework for the first clinical testing of our gene therapy strategy.

779. Glioblastoma Eradication by Combination Oncolytic Immunovirotherapy and Immune Checkpoint Blockade

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Glioblastoma, the most common primary brain tumor in adults, is invariably fatal despite all current therapies, with a median survival of about 15 months. Glioblastoma contains cancer stem cells, a subpopulation of cells likely important for tumor initiation, progression, heterogeneity, recurrence and resistance to therapy, and thus critical targets for therapy. We used a mouse syngeneic glioblastoma stem cell (GSC) tumor model, 005, isolated from activated H-Ras and Akt induced, Tp53+/- gliomas in C57Bl/6 mice. 005 GSCs are highly tumorigenic and their tumors recapitulate the hallmarks of human glioblastoma. Oncolytic herpes simplex viruses (oHSVs) are genetically engineered to selectively replicate in cancer cells and not normal cells. Two intratumoral injections of oHSV expressing murine IL-12 (G47Δ-mIL12) significantly extended survival in mice bearing orthotopic 005 tumors, whereas G47Δ-empty had a minimal effect.

Immune checkpoints, such as CTLA-4 and PD-1 play a critical role in regulating immune responses and suppressing immune effector cells. PD-1 and CTLA-4 are expressed predominantly on T-cells, while PD-L1 is also expressed on endothelial and tumor cells. Blocking antibodies to these molecules are effective at reversing tumor-induced immunosuppression. Therefore, we hypothesized that an IL12-expressing oHSV, which induces antitumor immune responses, should synergize with checkpoint inhibitor antibodies in inhibiting glioblastoma growth.

Mouse 005 GSCs are relatively non-immunogenic, lacking MHCI and MHCII expression, with PD-L1 only expressed on a minority of 005 GSCs *in vitro*, making them an excellent immunocompetent glioblastoma model. MHCI and PD-L1, but not MHCII, can be induced by IFNγ in almost all 005 GSCs. Mice with established orthotopic 005 GSC-derived tumors were treated with a single intratumoral injection of G47Δ-mIL12 followed by systemic anti-mPD-1, anti-mPD-L1, anti-mCTLA-4, or isotype control antibodies. Single treatments minimally, but significantly ($p \leq 0.05$), improved median survival compared to mock, while the combination of G47Δ-mIL12 and single antibodies further extended survival modestly. The limited efficacy of the dual combination was not due to the inability of antibodies to cross the blood brain/tumor barrier, since antibodies were detected within the brain tumor. Significantly, the triple combination (G47Δ-mIL12+anti-PD-1+anti-CTLA-4) was synergistic and cured 89% of mice ($p \leq 0.0001$ vs. oHSV plus single antibody). The cured mice were protected from lethal tumor re-challenge 6 months after treatment. We have examined the immune cells infiltrating the 005 tumors that are associated with this response. Depletion studies demonstrated the necessity for multiple immune cell types. In this representative immunocompetent GSC model, the combination of intratumoral G47Δ-mIL12 and two systemic checkpoint inhibitors was necessary to eliminate established tumors. This triple combination should be translatable to the clinic and other immunotherapy-minimally responsive tumor types.

780. Understanding and Exploiting Immune Checkpoint Blockade in Animal Models of Gene and Viroimmunotherapies

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The use of immune checkpoint blockade (ICB) has shown great promise in a proportion of patients in which there is evidence of a pre-existing anti-tumor immune response. In this subset of patients, the anti-tumor T cell response appears to be functionally exhausted, as characterised by the expression of immune checkpoint molecules such as PD-1 and TIM-3 and the activation of transcriptional programmes which indicate chronic exposure to antigen and dysfunctional T cell activation. However, most animal models of tumor growth and treatment do not mimic this situation in that there is rarely a pre-existing anti-tumor T cell response in place and the T cell population is not functionally or phenotypically exhausted. For example, we have shown that intravenous treatment with VSV rapidly expanded a population of effector-differentiated ($CD44^{hi}$ $CD62L^{lo}$) virus specific T cells. A vast majority of these cells were double positive for PD-1 and TIM-3, as would be expected of an acutely activated population of T cells responding to an infection. However, we showed that treatment with ICB was usually only marginally successful in enhancing the efficacy of the virotherapy itself. In addition, in a model of combined oncolytic viroimmunotherapy and adoptive T cell therapy, ICB was completely ineffective at enhancing anti-tumor therapy despite an elevated proportion of $CD8^+$ $PD\text{-}1^+$ $TIM3^+$ effector cells induced by the co-administration of VSV. Based on these data, we conclude that the animal models used to test our immunotherapies represent immunological scenarios distinct from what is present in patients who have truly exhausted T cell phenotypes. Therefore, we have developed improved models in which mice become immunologically tumor experienced through prior exposure to effectively treated tumors. Hence, mice which underwent complete regression of established subcutaneous tumors following suicide gene immunogenetherapy, adoptive T cell therapy, or oncolytic viroimmunotherapy, became highly sensitive to ICB upon tumor recurrence especially if recurrence was prematurely induced by immune stimulation. In addition, ICB delivered to tumor experienced/cured mice was particularly effective in promoting rejection of tumor rechallenge in combination with neo-antigen expressing gene modified vaccines. These data suggest that animal models in which the immune system has been primed with tumor rejection antigens will more faithfully mimic the clinical

situation in which ICB is effective. Moreover we demonstrate that boosting a pre-existing weakly anti-tumor T cell population with novel therapies which promote neo-epitope spreading will be effective in combination with ICB.

781. CART Cells Secreting IL18 Augment Antitumor Immunity and Increase T Cell Proliferation

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It is well established that T cell receptor (TCR) engagement and costimulatory signaling provide the critical signals that regulate T cell activation, proliferation and cytolytic functions. In addition to TCR and costimulatory signals, exogenous cytokines also play a fundamental role in modulating T cell function, as reflected in the influence of cytokines produced by CD4+ T helper cells on the activity of CD8+ cytotoxic T cells. Although many immune cytokines have been evaluated for their ability to promote anti-tumor immunity when delivered exogenously, reports of toxicity and/or lack of efficacy have limited their success. Several attempts to replace exogenous cytokine treatment with T cell-autonomous cytokine production have been reported. For example, IL12-expressing T cells have been investigated in multiple preclinical studies, and IL12-expressing tumor infiltrating lymphocytes (TIL) were recently used to treat melanoma patients. Although IL12-expressing TIL therapy resulted in effective anti-tumor response, toxicity from persistent, elevated serum IL12 concentrations was observed. We hypothesized that including an IL18 gene expression cassette in the CAR lentiviral vector might overcome the requirement for exogenous cytokine stimulation, and promote improved antitumor CART cell effects. IL18 was initially characterized as an inducer of interferon-γ (IFNG) expression in T cells, and was shown to activate lymphocytes and monocytes, without eliciting severe dose limiting toxicity in a previous clinical study. The combination of IL18 with IL12 treatment promotes Th1 and NK cell responses, but also induces lethal toxicity in murine models, reflecting the dynamic tension between achieving high potency and inducing toxicity in cytokine based immunotherapies. Despite initial indications that IL18 treatment alone may be only minimally effective in boosting anti-tumor immunity, we previously reported that recombinant human IL18 could significantly enhance engraftment of human CD8 T cells in a xenograft model. In the current study, we demonstrate that CART-cell autonomous IL18 expression promotes significant T cell proliferation *in vitro* and *in vivo*. Moreover, IL18-CART exhibited enhanced cytotoxicity and cytokine production *in vitro*. Importantly, we demonstrated that IL18-expressing CD19 CART cells exhibited remarkable proliferation and persistent antitumor activity against CD19-expressing tumor cells *in vivo*. These data support the utility of engineering CART cells to express IL18. We further developed an inducible expression system that couples IL18 expression levels to CAR stimulation, thereby reducing potential toxic effects of persistent IL18 expression in the absence of tumor antigen. Collectively, these studies identify a novel approach to enhancing CART cell expansion *in vivo*, which is a critical determinant of successful CART cancer immunotherapy.

782. Improving CAR-T Cell Therapy with Oncolytic Virus-Driven Production of Bispecific T-Cell Engager

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T cells modified to express a chimeric antigen receptor (CAR-T) have shown significant promise in clinical trials to treat B cell malignancies but have more limited efficacy in solid tumors. The immunosuppressive tumor microenvironment often results in poor T cell infiltration and proliferation *in vivo* followed by T cell exhaustion. Oncolytic viruses may have a dual benefit in combination with T cells of debulking the tumor to allow better T cell infiltration and of creating a danger signal to overcome the immunosuppressive environment and spur T cell activation. Here, we hypothesized that an oncolytic virus armed with an EGFR-targeting bispecific T cell engager (BiTE) would further improve CAR-T cell therapy by: (i) redirecting CAR-negative untransduced (UTD) T cells (commonly present in CAR-T preparations infused to patients) to cancer cells and (ii) minimizing tumor immune evasion by antigen loss due to targeting of two distinct tumor antigens.

In this project, an E1a-Δ24-based oncolytic adenovirus (ICOVIR-15K) modified to express a BiTE targeting EGFR (ICOVIR-15K-cBiTE) was combined with CAR T cells targeting Folate Receptor alpha (FRa) antigen (Mov19-ICOSz CAR-T).

The cytotoxicity of the therapy was tested *in vitro* through coculture experiments with tumor cells and analyzed using the xCELLigence real-time cell analysis instrument. The combination therapy composed of CAR-T cells and ICOVIR15K-cBiTE significantly improved cytotoxicity compared to each monotherapy and resulted in complete clearance of each of the tested tumor cell lines. The combination therapy also improved T cell activation and proliferation and resulted in increased cytokine production by both UTD and CAR-T cells. In contrast, the addition of the parental non-armed virus, ICOVIR-15K, showed a moderate improvement in cytotoxicity and T cell proliferation and no improvement in T cell activation or cytokine production.

To examine the effect of the combination therapy *in vivo*, NSG mice bearing HCT116, SKOV3 or Panc-1 tumors expressing FRa and EGFR were treated with ICOVIR-15K-cBiTE (intratumorally) and CAR-T cells (intravenously) alone and in combination. CAR-T therapy alone showed moderate anti-tumor effect in all three models and oncolytic virus alone showed some tumor regression in the slower-growing Panc-1 and SKOV3 models. However, the combination therapy resulted in significantly decreased tumor growth and increased survival compared to each element alone in all three models.

Overall, these results demonstrate that the combination of a BiTE-secreting oncolytic adenovirus with adoptive CAR-T cell therapy overcomes key limitations of both modalities as monotherapies for solid tumors.

783. Gene Modification of Human Hematopoietic Stem Cells with Suicide Genes as a Safety Control System

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Background: Gene-modified human hematopoietic stem cells (HSC) have been used to introduce genes to correct metabolic or cellular disorders or to prevent or treat diseases. We propose gene-modification of HSC to enable persistent generation of multilineage immune effectors able to directly target cancer cells. Concerns regarding malignant transformation, abnormal hematopoiesis and autoimmunity exist, making the co-delivery of a suicide gene a necessary safety measure. We have compared two suicide gene systems: the herpes simplex virus thymidine kinase (HSV-sr39TK) and a truncated epidermal growth factor receptor (EGFRt). To enable future applications, those transgenes were co-delivered with anti-CD19 chimeric antigen receptor (CAR). We hypothesize gene-modified HSC can be successfully ablated using these suicide gene systems. **Methods:** Third generation self-inactivating lentiviral vector constructs were used to co-deliver an anti-CD19 CAR and HSV-sr39TK or EGFRt. Efficacy was tested using cytotoxicity assays. Jurkat and primary cells expressing HSV-sr39TK were incubated with varying concentrations of ganciclovir. For EGFRt, an antibody-dependent cell-mediated cytotoxicity (ADCC) assay was used with the target cells incubated with leukocytes and the monoclonal antibody cetuximab. For both assays, the cells were stained and analyzed by flow cytometry to determine the percentage of surviving cells. For *in vitro* assays, gene-modified HSC were differentiated into myeloid cells over ten days to allow transgene expression before cytotoxicity challenges. Gene-modified HSC were also engrafted into immunodeficient NSG mice for *in vivo* experiments using treatments with intraperitoneal ganciclovir 50mg/kg/day over 5 days or intraperitoneal cetuximab 1mg/mouse/day over 12 days. Persistence of gene-modified cells was assessed by flow cytometry and ddPCR of animal tissues. **Results:** Human cells had decreased rates of survival for the HSV-sr39TK transduced cells when incubated with varying concentrations of ganciclovir, as compared to controls ($p<0.0001$), with remaining survival of gene-modified cells of 15% in the assays employed. For the EGFRt transgene, cytotoxicity was significantly increased ($p<0.0001$) in target cells expressing EGFRt after 4-hour incubation of leukocytes with target cells and cetuximab 1μg/mL, compared with either EGFRt+ cells without cetuximab, and non-transduced cells with or without cetuximab. This was seen at all effector to target ratios and average remaining gene-modified cells approached 10% in the assays employed. Mice humanized with gene-modified HSC presented significant ablation of gene-modified cells after treatment with either ganciclovir for the HSV-sr39TK transgene ($p=0.05$), or cetuximab for the EGFRt transgene ($p=0.002$). Remaining detected gene-modified cells in both models were close to background on flow cytometry and within two logs of decrease of vector copy numbers by ddPCR in mouse tissues. **Conclusions:** Ganciclovir-mediated killing of HSV-sr39TK transduced cells was shown to be effective in cells differentiated from gene-modified human HSC. Cetuximab ADCC of EGFRt-modified cells also determined effective killing. These results give proof of principle for CAR-modified HSC regulated by suicide gene, and further studies are needed to enable clinical translation of this approach. Different ablation approaches, such as inducible caspase 9 or co-delivery of other inert cell markers should be evaluated.