

Programmable Molecular Scissors: Applications of a New Tool for Genome Editing in Biotech

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Targeted genome editing is an advanced technique that enables precise modification of the nucleic acid sequences in a genome. Genome editing is typically performed using tools, such as molecular scissors, to cut a defined location in a specific gene. Genome editing has impacted various fields of biotechnology, such as agriculture; biopharmaceutical production; studies on the structure, regulation, and function of the genome; and the creation of transgenic organisms and cell lines. Although genome editing is used frequently, it has several limitations. Here, we provide an overview of well-studied genome-editing nucleases, including single-stranded oligodeoxynucleotides (ssODNs), transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and CRISPR-Cas9 RNA-guided nucleases (CRISPR-Cas9). To this end, we describe the progress toward editable nuclease-based therapies and discuss the minimization of off-target mutagenesis. Future prospects of this challenging scientific field are also discussed.

In modern biotechnological and medical research, tools for precise and predetermined genome modification are used to identify genes with diverse functionality. To study the functions of genes, small interfering RNAs (siRNAs) are widely used to suppress gene expression. However, the use of siRNA-mediated knockdown is questionable because its threshold success rate is below 70%.¹ This method is hindered by several issues, including low effectiveness of engineered constructs at the chromosomal target, time-consuming processing, labor-intensive selection criteria, and possible undesirable mutagenic effects.² Although siRNA is useful to study gene function, the knockdown deficiency often occurs. siRNA-mediated knockdown offers temporary inhibition of gene function, which limits our ability to correlate phenotype and genotype. In the early stages of molecular biotechnology, genes were categorized by features of their mutant phenotype. Subsequent development of genome editing is more advantageous than the siRNA technology for assessing the function of a gene or genotype.^{2,3}

Currently, in addition to siRNA technology, modified DNA-binding proteins, such as zinc-finger proteins and transcription activator-like effectors (TALEs), have been widely used for sequencing target DNA and for gene regulation.³⁻⁵ In modern biotechnological research, the

methods for gene modification primarily use three types of endonucleases: zinc-finger nucleases (ZFNs), TALENs, and CRISPR-Cas9. Recently, the use of CRISPR-Cas9 has increased tremendously compared with that of other endonucleases (e.g., ZFNs and TALENs); the latter are not used as frequently because of several drawbacks, such as single-site targeting, occurrence of nonspecific mutations, and low efficiency.⁶⁻¹⁰ Single-stranded nucleotide sequences with a complementary sequence of approximately 20 pairs have been designed to target a desired region in the genome.^{11,12} These three types of endonucleases are based on similar mechanisms in modification of the genome, cleaving chromosomal DNA in a specific location for targeted alteration of the genome.¹³

Site-specific programmable nucleases can generate DNA double-strand breaks (DSBs),¹⁴ which can lead to 2-fold increases in homologous recombination.¹⁵ Therefore, site-specific programmable nucleases can be employed in targeted mutagenesis.¹⁶ There are two main mechanisms underlying DSB repair: non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ, the principal DNA repair mechanism found in eukaryotes, repairs DSBs by ligating the broken ends of DNA. This ligation is mediated by specific protein factors that re-connect the strand without a homologous DNA template.^{17,18} There are two major pathways in NHEJ: (1) direct ligation of two ends of the targeted DNA, or (2) end-joining via deletion or insertion of nucleic acids. In both of these events, the occurrence of NHEJ-mediated deletions and mutations are well documented.^{19,20} In contrast, HDR can also be implemented to repair DSBs, but requires a homologous DNA template (Figure 1).²¹⁻²⁴ These mechanisms, however, cannot be used to implement a gene knockin into non-dividing cells.²⁵ Hence, a

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Genome engineering by ZFNs, TALENs or CRISPR/Cas9

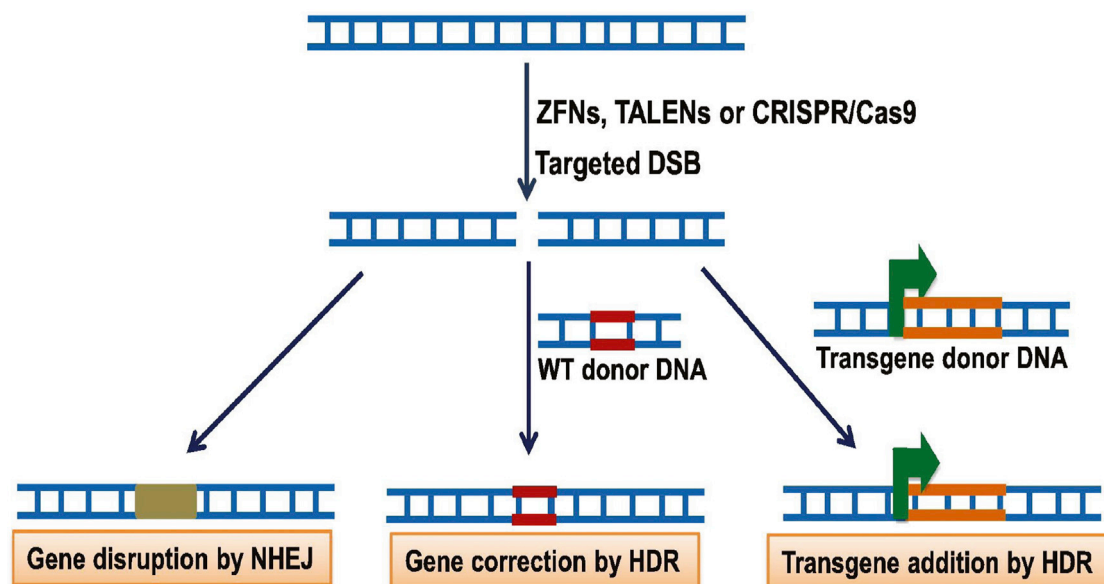


Figure 1. Genome Engineering Using Programmable Nucleases

Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-Cas9 are used to induce targeted double-strand breaks (DSBs) at the desired chromosomal locus. Non-homologous end joining (NHEJ) or homology-directed repair (HDR), one of the two cellular repair pathways, is then used to repair the DSB. NHEJ can be used to knock out genes, whereas HDR can be used either for gene correction or to introduce precise alterations into the genome; this is directed by a homologous DNA template. Adapted from Chandrasegaran and Carroll²² and Ramalingam et al.,²³ copyright (2015) Creative Commons Attribution 4.0 International.

CRISPR-Cas9-based method, known as homology-independent targeted integration (HITI), was developed for this purpose. Because HITI does not require a homologous arm, it has a recognition site for flanking Cas9 so that Cas9 can cut both target and donor sequences. The donor then integrates in the genome and repairs DSBs in an independent pathway similar to that of NHEJ. This technique has allowed integration of a gene into non-dividing cells with only 2.1% insertion in the opposite direction.²⁶

In early 2012, CRISPR-Cas9 nucleases were developed as a tool for modifying the genomes of various cell lines and those of several living organisms.²⁷ For DNA modification, CRISPR-Cas9 systems outperform the TALEN and ZFN systems.^{10,28–30} In addition to its editing capability, the CRISPR-Cas9 system possesses curative potential³¹ and can be used to correct gene mutations in various genetic diseases such as β -thalassemia, cystic fibrosis, hemophilia, Duchenne muscular dystrophy, and hereditary tyrosinemia type I.^{32–39} Systems based on ZFNs, TALENs, and CRISPR-Cas9 have been used to alter the genomes of plants, animals, and even humans. These tools are now mainly used to alter the desired sequences in specific genes and have impacted numerous fields, such as biopharmaceutical development; gene surgery; alteration, regulation, and function of genome structure; and production of biofuel, food, and transgenic cell lines and animals (Figure 2). Although commonly used for genome alteration, these tools still have numerous issues.

In this review, we provide an overview of common genome-editing nucleases including ZFNs, TALENs, CRISPR-Cas9, and single-stranded oligonucleotides (ssODNs). These tools are used for genome editing in various fields of cellular and molecular biotechnology, including genome alteration in cells and embryos, development of biocompatible drugs, targeting of genetic diseases, and development of traits in plants or farm animals. Finally, we discuss the prospect of using genome editing for editable nuclease-based therapies and for limiting off-target mutagenesis. We also discuss the future prospects of these technologies, including their use in genome editing and modeling specific diseases.

Origin of Programmable Nucleases

Although double-strand breaks in the DNA cause severe fatal effects in cells, DNA breaks can be repaired by NHEJ and HDR.¹⁵ Gene targeting by HDR is not an efficient mechanism in higher eukaryotes. One in a million treated cells may undergo this kind of genome modification.²¹ Molecular scissors, such as restriction endonucleases, cannot induce a DSB at a targeted chromosomal location. This is because they can only identify very short DNA templates of 4–8 bp. Inducing a targeted DSB is a major problem when employing HDR technology for genome engineering of eukaryotic cells.²² This led to the development of a generalized delivery system to target genomic DSB at a distinctive chromosomal locus and to help stimulate HDR.²²

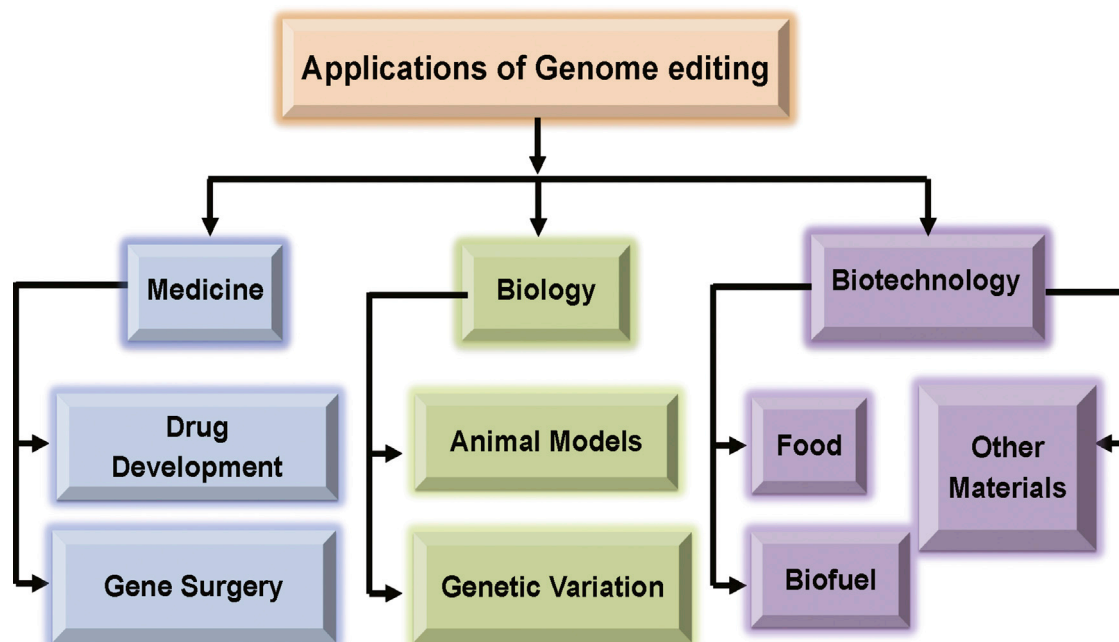


Figure 2. An Overview of Applications of Genome Editing

Genome editing can be used in various fields of biotechnology, including biopharmaceutical development and gene therapy; genome structure alteration, regulation, and function; and production of biofuel, food, and transgenic cell lines and animals.

Numerous options, such as programmable nucleases, ZFNs, TALENs, and RNA-guided CRISPR-Cas9, have been used to induce a targeted DSB. These programmable nucleases possess several common features, which can be used in the editing of eukaryotic genomes. Using a rare-cutting endonuclease, DNA DSBs can be repaired via homologous recombination, which can improve the repair by 2- to 3-fold efficiency in the targeted region. Conversely, DSBs can also be repaired by NHEJ in the absence of a gene-marking vector or homologous donor DNA.^{16,40} Programmable nucleases for NHEJ- or HDR-mediated repair of site-specific DSBs are commonly used for targeted genetic modifications, gene disruption, gene insertion, gene correction with point mutagenesis, and chromosomal rearrangements.^{13,41}

Designed Nucleases

ZFNs

Zinc-finger (ZF) motifs were first described by Klug et al.⁴² as zinc-binding domains in transcription factor IIIA in *Xenopus oocytes*; ZFs occur more frequently in eukaryotes than in prokaryotes.⁴² Every ZF is composed of approximately 30 amino acids in a conserved $\beta\beta\alpha$ configuration, where the zinc atom is attached to two pairs of cysteine and histidine residues. Furthermore, each ZF is combined with DNA via an α -helix introduced into the main channel of the DNA double helix, and by a recognized 3- to 4-bp sequence. Generally, most ZFs are attached to a 3-bp target template and then change the recognition site to a 4-bp cross-strand. This mechanism influences the specificity of neighboring ZFs, thereby complicating the generation of ZF proteins (ZFPs). ZFP generation is mediated by a simple modular design

because all ZFs identify a triplet sequence (Figure 3A).²² To generate DNA-binding ZFPs, three to six ZFs are linked together in a tandem sequence (Figure 3A).²² ZFPs are generally composed of tandem arrays of C_2H_2 zinc fingers, which are mainly responsible for sequence specificity of ZFNs.⁴³ Altering DNA-binding specificities of ZFNs is an important feature in construction of a programmable nuclease; this is due to mutagenesis that can occur in ZFNs.^{44,45}

ZFNs are generally composed of two important domains: the DNA-binding ZF protein (ZFP) domain and the nuclease domain derivative from the FokI restriction enzyme.⁴⁶ A type IIS restriction enzyme, FokI is usually used to identify non-palindromic pentadeoxy-ribonucleotide in the DNA double helix. FokI cleaves the nucleotides downstream of the recognition site. The functionality of FokI is regulated by two distinguishable protein domains: (1) domain F_R and (2) domain F_N (Figure 3B).²² The former is attached to a specific recognition site in the DNA to help propagate a signal to the latter via allosteric interaction. Upon receiving the signal, the latter activates the endonuclease activity of FokI after which the final cleavage is induced (Figure 3B).²² The modular characteristics of FokI endonuclease were confirmed by crystal structures of local FokI and DNA-binding FokI; the cleavage domain was isolated from the recognition domain to prevent any contact with DNA.^{47,48} Nevertheless, ZFNs produced by traditional methods often showed limited DNA targeting activity and cytotoxicity when off target.^{49,50} Studies on ZFNs have confirmed that, on average, a single functional ZFN pair can be generated per 100-bp DNA sequence,⁵¹ implying that ZFNs may allow efficient genome editing.

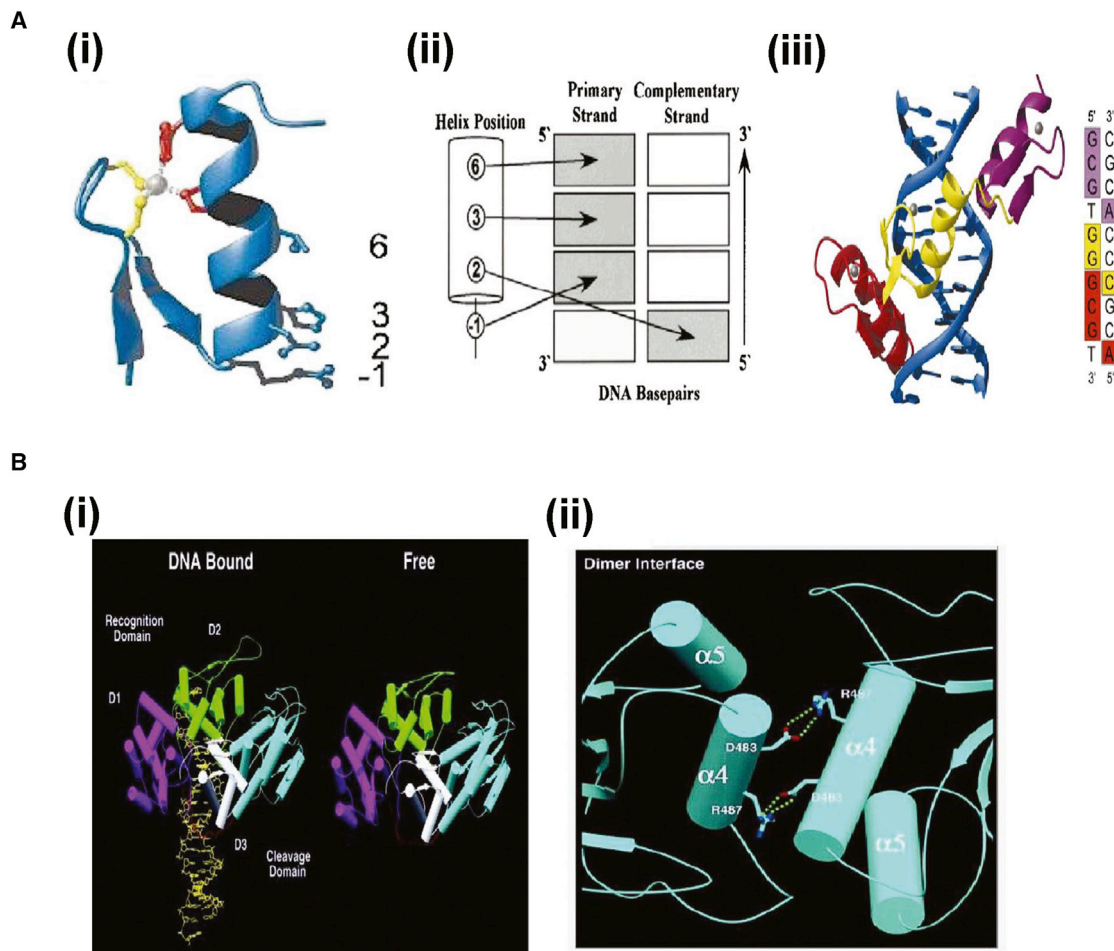


Figure 3. Illustration of DNA Recognition by ZFPs and Crystal Structures of FokI and How FokI Bound to DNA

(A) DNA recognition by ZFPs. (Ai) Structure of a single ZFN, (Aii) DNA recognition by ZFNs, and (Aiii) structure of three-finger Zif268 bound to its cognate site. (B) Crystal structures of FokI and FokI bound to DNA. (Bi) Structures of FokI-DNA complex and of FokI enzyme alone. In both structures, the FokI cleavage domain piggybacks on the recognition domain. (Bii) Native FokI crystallizes as a dimer. The dimer interface is at the FokI nuclease domain, which is formed by two salt bridges between arginine (R) and aspartic acid (D) residues of the FokI monomers. Reproduced from Chandrasegaran and Carroll,²² Wah et al.,⁴⁸ Miller et al.,³⁰⁰ and Pavletich et al.,³⁰¹ copyright (2015) Creative Commons Attribution 4.0 International.

Different types of computer-based programs are currently available for searching possible ZFN target sites in a certain DNA sequence. Among such programs, PROGNOS (<http://bao.rice.edu/Research/BioinformaticTools/prognos.html>) and ZiFiT (<http://zifit.partners.org/ZiFiT/>) are most commonly used.⁵² In addition, EENdb (<http://eendb.cbi.pku.edu.cn/>) can be used along with other databases.⁵²

TALENs

TALENs are simple modular codes for DNA recognition processed by the TALE proteins. TALEs are naturally occurring proteins commonly found in *Xanthomonas*, a pathogenic bacterium that infects plants.^{53,54} TALENs can act as a versatile platform for programmable DNA-binding proteins. There are several similarities between TALENs and ZFNs. For example, similar to ZFNs, a FokI nuclease domain is also found in TALENs. Moreover, TALENs utilize different

types of DNA-binding domains, mostly isolated from *Xanthomonas* spp.¹³ The central domain of a TALE consists of a repeating unit composed of approximately 34 amino acids. Each of these units is recognized as a single base pair. The specificity of TALEs mainly depends on the two hypervariable amino acids at the 12th and 13th positions, which are called repeat variable diresidues (RVDs).^{55,56}

Four different RVD amino acid diresidues (i.e., NI, HD, NG/HG, and NN) are mainly used to identify adenine (A), cytosine (C), thymine (T), and guanine (G)/adenine (A), respectively. This recognition process of TALE modules appears to function independently from neighboring modules, unlike the mechanism used by ZFPs. The presence of a DNA recognition code mainly provides close interaction between the array of amino acid repeats and the nucleotide sequence of a genome; therefore, new TALENs can be blueprinted with desired



sequence specificities.^{57,58} The modular character of TALE patterns combined with the simple DNA detection code makes TALENs suitable for constructing common nucleases.⁵⁹ Similar to programs used to design ZFNs, web-based computer programs, such as E-TALEN (<http://www.e-talen.org/E-TALEN/>),⁶⁰ are also available for designing TALENs.

In mammalian genomes, many types of complex TALENs can also target distinctive loci. TALENs are generally simpler to construct than are ZFNs. However, the designed sequences for encoding TALENs are much longer than those for ZFNs. The TALE and ZFN motifs are similar in size; however, TALE motifs recognize only one base, whereas ZFNs recognize 3–4 bp sequences. These consensus series of TALE motifs with highly repetitive sequences increase the complexity of TALEN-encoding genes in *E. coli*,²² presenting a challenge in increasing the numbers of these genes. These TALEN-encoding genes also complicate virus-mediated delivery into mammalian cells. To overcome those challenges, different types of effector domains can be combined with TALE repeats for targeted genetic alterations.

Cloning DNA segments that encode TALE arrays involves certain technical challenges posed by large similar repeat sequences, requirement of approximately 20 RVDs, and increased consumption of cost.⁶¹ Numerous techniques for rapid assembly of custom TALE arrays include: Golden Gate cloning systems,⁶² solid-phase assembly,^{59,63} and ligation-independent cloning.⁶⁴ All of these systems are currently used for high-throughput sequencing. In brief, Golden Gate cloning systems utilize a type IIS restriction enzyme for producing four-base extensions at individual DNA fragments encoding RVD modules. DNA fragments with complementary extensions can be linked to generate numerous RVD elements in a planned array.^{65,66} Any DNA sequences can be targeted by TALENs, which is the main advantage of this type of nuclease over other types. Small DNA sequences can be mutated using TALENs, which cannot be accomplished by ZFNs or RNA-guided engineered nucleases (RGENs). The one limitation for the synthesis of TALENs is that the 5' ends of binding sites, which start with thymine (T), are recognized by two amino acid terminal folds.⁵⁵

To overcome this thymine-specific recognition, extensive research has focused on TALE variants. Such efforts have made it possible to help recognize bases other than thymine at the 5' end. This reorganization process has been useful for increasing the range of sites targetable by TALENs.^{67,68} The production of customized TALENs or genome-modified cell lines has been pursued actively by commercial organizations such as Collectis Bioresearch (Paris, France), Transposagen Biopharmaceuticals (Lexington, KY, USA), and Life Technologies (Grand Island, NY, USA).³

CRISPR-Cas9

A number of in-depth reviews have covered adaptive immunity in bacteria, which involves the mechanism of adaptive resistance based on the CRISPR-Cas system.^{69–77} Bacteria and archaea commonly use

this mechanism to degrade complementary sequences that are present in viral and plasmid DNA.⁷⁸ These organisms generally capture DNA fragments of approximately 20 bp from viruses and plasmids, which facilitates the formation of a CRISPR sequence. These sequences, referred to as protospacers, can be inserted into their own genome. Pre-CRISPR RNA is transcribed from CRISPR regions of type II CRISPR systems belonging to two major classes. Pre-CRISPR RNA is then processed to form a short CRISPR RNA (crRNA). This process is facilitated by the transcription of *trans*-activating crRNAs (tracrRNAs), which are combined with crRNAs.⁷⁹ The crRNA-tracrRNA complex ultimately associates with Cas9 to form an active endonuclease for the degradation of foreign DNA; this active endonuclease is called dual RNA-Cas9.⁷⁸ RNA-Cas9 targets a 23-bp sequence that consists of a guide sequence (20-bp) in crRNA and the 5'-NGG-3' sequence identified as the protospacer-adjacent motif (PAM).⁸⁰

The crystal structure of Cas9 proteins, derived from different species, can exist alone as an inactive form, although it can become active through the binding of a single-chain guide RNA (sgRNA).⁸¹ A new RGEN-encoding plasmid can be easily prepared by cloning DNA sequences that encode either crRNA or sgRNA into a suitable vector. This process is much simpler than complicated protein-engineering methods for the synthesis of RGENs.^{82–84} Due to a basic design and simple preparation methods, RGENs are more suitable for targeting endogenous loci in human cells than are ZFNs and TALENs. In the case of RGENs, only RNA needs to be designed, rather than protein-engineering two nucleases to recognize the target sequence. The CRISPR-Cas9 system is more advantageous for new targets because it can target many objects simultaneously; this system has been widely used in research laboratories worldwide. Moreover, the CRISPR-Cas9 system is economical for small laboratories, making it a reasonable choice for genome engineering procedures.²² In addition to its utility in editing genomic sequences, the CRISPR-Cas9 system is a non-mutagenic gene regulation tool that is completely sequence specific. Several companies, such as Sigma-Aldrich, System Biosciences, ToolGen, and Transposagen Biopharmaceuticals, commercially offer RGENs for laboratory use.

ssODNs

Engineering the mammalian genome is a powerful genetic approach for developing novel therapeutics for treating hereditary diseases. In addition to complete gene knockout, there is also single-nucleotide exchange for re-engineering of mammalian genomes via ssODNs. This method has been used to generate nucleotide changes and often works in combination with other genome-editing tools.^{85–88} The lower efficiency of ssODNs makes them incompatible for therapeutic application. However, adjuvants can be used to enhance the frequency of ssODNs. The overall activity of genome editing by ssODNs can be improved approximately 5- to 10-fold by inducing double-strand DNA breaks before introducing ssODNs.^{89–92} TALENs have also been used in combination with ssODNs to exchange single nucleotides at specific sites in a gene.^{66,93–95} To repair single-point mutations, combining TALENs with ssODNs is used to generate substantial changes at both genotypic and phenotypic levels.^{95–97}



The core mechanism of single-point mutation repair involves reduction of ssODNs by TALENs, which are required for gene editing of a specific entry site provided for the oligonucleotide. An oligonucleotide with an adjacent stretch of RNA and DNA has been developed for correction of a single mutation in episomal and chromosomal targets in mammalian cells.^{98,99} This type of oligonucleotide is capable of correcting a site-specific mutation in tissue-cultured cells when administered *in vivo*. This stable and steady process of gene correction, mediated by an RNA-DNA oligonucleotide, was established by clonal analysis at the sequence level.¹⁰⁰ This RNA-DNA oligonucleotide may be a promising curative approach for genetic diseases. To inhibit the expression of a desired gene, ODNs can be used as antisense oligonucleotides that hybridize with target mRNAs complementary to the sequence of the antisense oligonucleotide.¹⁰¹

In mammalian cells, a 40-nt ssODN is sufficient for genetic modification.^{102,103} In yeast, however, 20–70 bases are needed to induce modifications. A plasmid vector, bearing a mutant neomycin phosphotransferase (*NPT*) gene, can be co-transfected with a 40-base single-stranded oligomer that comprises the sequence of wild-type *NPT* in mammalian cells. Cells containing *NPT* are selected by G418 antibiotic screening. These cells contain wild-type DNA molecules that result from recombination between two DNA molecules. This method is advantageous in altering or introducing point mutations into the genomes of human cells.¹⁰² In yeast, sense oligonucleotides generate nearly 20-fold more transformants than do antisense oligonucleotides. This discrepancy was confirmed with oligonucleotides designed to create modifications at six different locations along the gene. The contrary result was likely caused by the length and sequence of the oligonucleotide, number of alterations, and host strain.¹⁰³ This implies that ssODNs can be used in various gene-targeting approaches including the development of transgenic animals, improvement of isogenic cell lines, site-specific mutagenesis, and gene therapy.

Applications of Genome Editing in Mammals

In life sciences research, biotechnologies that enable deletion, insertion, and modification of DNA sequences in cells or organisms allow us to evaluate specific gene functions. These genome-modification technologies have also enabled the examination and large-scale manipulation of genes and protein networks. In addition to enabling manipulation of transcriptional regulation at a specific locus, genome editing can reveal the mechanisms regulating genetic systems. In modern biotechnology, the underlying regulatory mechanisms of genetic building blocks are helpful for reverse engineering of useful biological systems. The concurrent modifications of multiple genes can have adverse effects that trigger complex polygenic disorders. Conversely, genome-editing technologies can be used to directly correct harmful mutations in humans.¹⁰⁴ Potential applications of programmable nucleases in humans are discussed below and overviewed in [Figure 4](#).

Genome Editing in Farm Animals

With the rapidly growing population, there is an urgent need to develop biotechnological approaches that enhance animal production, while reducing the risks associated with climate change and/or

environmental pollution. In the recent past, genotyping and whole-genome sequencing improved our ability to analyze the genetics of farm animals. Production of genetically edited pigs was a milestone in genome manipulation of farm animals.¹⁰⁵ In recent years, genome editing has been performed via deletion, addition, and modification of base pairs at targeted loci. All of these genomic alterations are permanent and heritable in the following generations of livestock. Similar to the process occurring in plant species, ZFNs, TALENs, and CRISPR-associated endonuclease Cas9 (CRISPR-Cas9) are universally used to modify gene functions via NHEJ.^{106–108} However, insertions and additions via NHEJ can vary in size and sequence, making screening of nonfunctional clones more difficult.¹⁰⁹ Specific genes of interest in the genomes of cattle, sheep, and pigs have been edited to yield viable zygotes and living animals. *MSTN*, the gene encoding myostatin, can generate more extensive muscling in cattle, sheep, and pigs, whereas the *POLLED* allele in cattle is commonly targeted for editing along with protein-encoding genes such as *RELA*.^{110–115} Editing of the *CDI63* gene for resistance to the porcine reproductive and respiratory syndrome (PRRS) in pigs is a recent achievement and has been used extensively in the production of PRRS-resistant pigs.^{116,117}

Using ZFN for genome editing begins with the introduction of plasmid DNA or mRNA encoding ZFN into the target cells or embryos via microinjection or transfection.¹¹⁸ Then the translated ZFN protein binds to its specific target sequence and cleaves the target DNA by activating FokI nucleases at 30°C. The binding of ZFN to targeted loci is slow at a lower temperature, which affects cell-cycle progression.¹¹⁹ Insertion of ZFN-encoding plasmid DNA or mRNA can potentially cause constant transcription, leading to nonspecific DNA cleavage. In the case of ZFN-encoding plasmid DNA, a temporary transfection protocol can be used to dilute the plasmid DNA; this is a major advantage of using ZFN. Alternatively, microinjection of ZFN-encoding mRNA is more accurate than transfecting with ZFN-encoding plasmid DNA.¹²⁰ Thus, microinjection of ZFN-encoding mRNA can reduce the risk for permanent assimilation of ZFNs, making ZFNs more efficient genome-editing tools than the conventional approach.

Recently, transgenic pigs have been used as alternatives to mouse models for evaluating human diseases and developing therapies; this is because symptoms of classical diseases in mice do not fully mimic those in humans. Pigs are more suitable models for investigating human diseases, such as cystic fibrosis, diabetes, and cancer, because pigs possess human-like genetic, anatomical, and physiological characteristics.^{121,122} Pigs are also vital organ donors in generating xenografts of human organs.¹²³ Knockout pigs, engineered using ZFNs, generally carry a hemizygous transgenic *EGFP* reporter allele. The endogenous porcine gene (such as peroxisome proliferators activated receptor- γ [*PPAR- γ*]) was the first gene successfully targeted by ZFN. These types of knockout pigs are useful in research on cardiovascular diseases.¹²⁴ Biallelic knockout live pigs are produced by using ZFNs to target an endogenous gene.¹²⁵ Furthermore, cattle with knocked out β -lactoglobulin (*BLG*), a major milk serum protein and allergen, have been produced via gene targeting with ZFN.^{126,127}

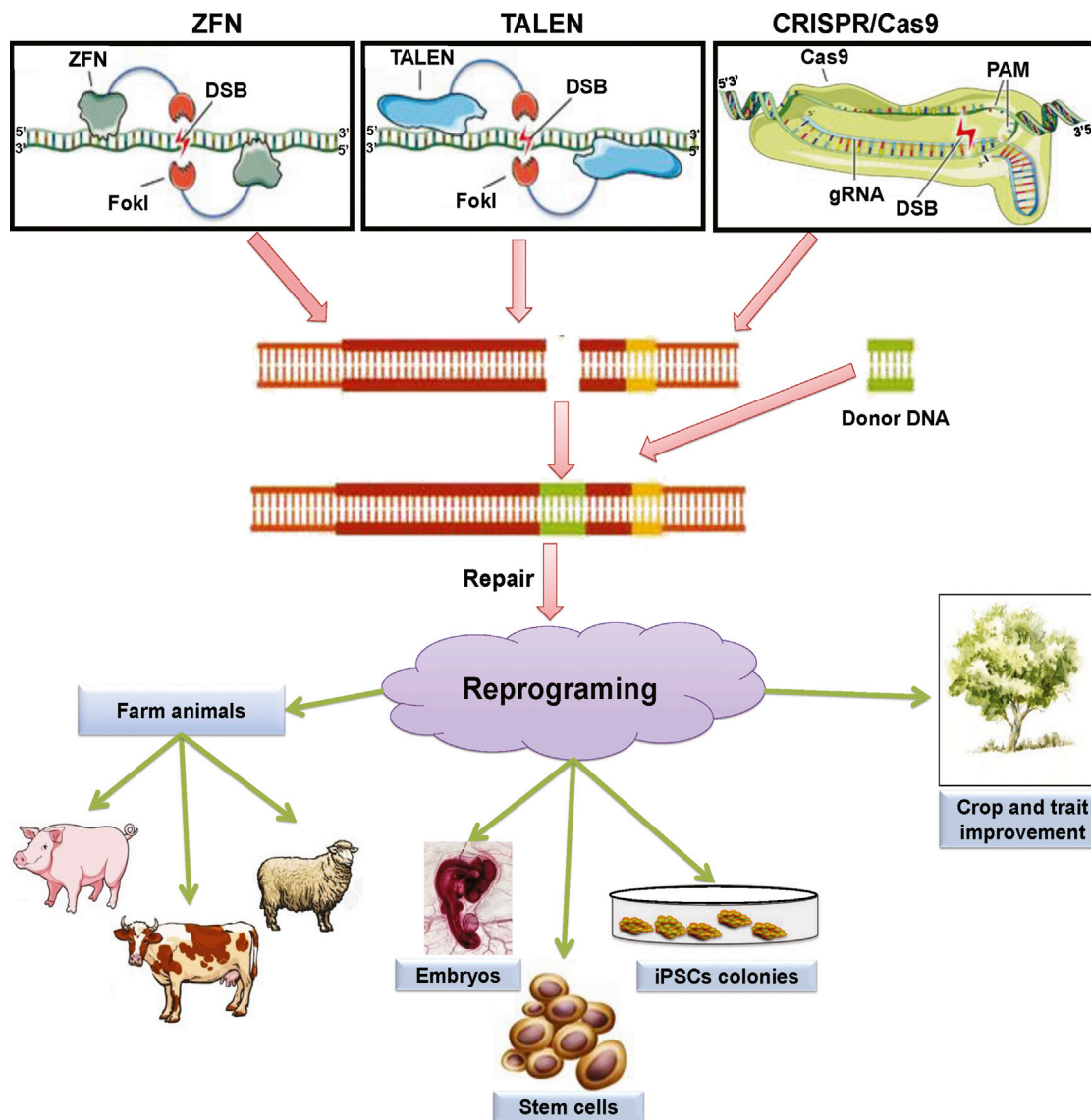


Figure 4. An Overview of Potential Applications of Programmable Nucleases

Programmable nucleases can edit the genome and reprogram genetic information, which consequently affects genome structure and function. This technology can be used to produce farm animals, transgenic cell lines (i.e., embryos, stem cells, and induced pluripotent stem cells [iPSCs]), and transgenic plants.

For knockout of the *BLG* gene, bovine fetal fibroblasts are transfected with ZFN mRNAs. Successful cell transfection is confirmed using the T7 endonuclease PCR to show that approximately 15% of the cells carry a mutated variant, whereas 3% are positive for the biallelic *BLG* gene knockout. The mutated *BLG* gene is also verified to detect the existence of any off-target mutagenesis. These studies indicate that specific genome editing with ZFNs in higher domestic animals results in less off-target mutagenesis than do other similar approaches.¹²⁶

The Gram-negative bacterium *Xanthomonas* is a plant pathogen that naturally produces TALEs. *Xanthomonas* infects numerous plant spe-

cies such as rice, citrus, cotton, pepper, tomato, and soybeans.¹²⁸ In cattle, two important genes in fibroblasts, viz. *ACAN* and *GDF8*, can be modified using TALENs. *ACAN* is responsible for congenital achondroplasia, whereas *GDF8* (growth differentiation factor 8/*MSTN*) acts as a controller of muscular growth. Bovine fibroblasts bearing the *GDF8* gene showed approximately 29% modifications when treated with TALENs.¹⁰¹ Conversely, the *ACAN* gene showed 77% modification after treatment with TALENs.¹⁰¹ To produce live progeny with desirable genetic modifications, customized cells are used for somatic cell nuclear transfer.¹¹⁰ Recently, TALENs were used to generate a porcine model of hypercholesterolemia by modifying the *LDL* receptor gene. TALEN-based alteration of the porcine



DMD gene was also developed for the model of Duchenne muscular dystrophy (DMD).¹¹⁰ To improve disease resistance in pigs, 20 ng/ μ L TALEN mRNA was recently introduced into porcine zygotes to target the *RELA* gene. Sixteen out of 56 porcine embryos were successfully transformed. The *RELA* gene is involved in tolerance to infection with the African swine fever virus.¹²⁹ Successful transformation was confirmed by DNA sequence analysis. Among the mutants, one-third was either homozygous or heterozygous. The genome sequences of the *MSTN* locus in both cattle and sheep show a high level of similarity; hence the same TALENs can be used in sheep and cattle.¹³⁰ To generate live progeny, TALEN mRNA was microinjected into ovine zygotes and then transferred into recipient ewes. After successful pregnancy and delivery, fewer offspring were found to have the heterozygous gene.¹³⁰

The specificity and efficacy of genome editing via CRISPR-Cas are similar to those achieved using ZFNs and TALENs. The von Willebrand factor (*vWF*) gene in pigs has been targeted by injecting Cas9 mRNA and sgRNA into zygotes. However, premature embryonic development was not affected by this process. After a successful pregnancy and delivery, the survival rate of piglets was approximately 88%. *In-vivo* piglet fetal development was not hampered by this genome modification.¹³¹ Other genes, such as the *p65* and the adenomatous polyposis coli (*APC*) gene loci, have also been targeted in porcine fetal fibroblasts by the CRISPR-Cas9 system.¹³² All of these endonucleases act as valuable gene-editing tools that are revolutionizing biological research and molecular medicine. To further expand this technology, we need to investigate model organisms and farm animals, develop biomedical models, and alter genes for the treatment of genetic diseases.

Genome Editing in Embryos

All the discussed programmable nucleases can also be used as genome-editing tools for human embryos. Altering genes in somatic cells at various clinical phases can be helpful in developing potential therapeutic applications. In recent years, CRISPR-Cas9 was used as a therapeutic tool to prevent the onset of β -thalassemia (a fatal blood disorder) by eliminating mutated human β -globulin (*HBB*) gene, which is responsible for the onset of β -thalassemia.¹³³ Nonetheless, this approach was not successful because of the ethical issues raised by the scientific community. To escape the ethical issues, it was decided to use “non-viable” embryos, which were tripronuclear zygotes generated by fertilization with two sperms. These non-viable embryos are commonly discarded in clinics.¹³⁴

In vitro fertilization may generate approximately 2%–5% polyspermic zygotes, which can produce blastocysts; however, this does not manifest in the *in-vivo* process.¹³⁵ Polyspermic zygotes are a model for assessing the target efficacy and off-target effects of CRISPR-Cas9.^{136,137} A subunit of adult human hemoglobin is coded by the β -globin (*HBB*) gene, which is mutated in β -thalassemia.¹³⁸ The location of the *HBB* gene on chromosome 11, within the β -globin gene group, consists of four other globin genes: *HBE*, *HBG2*, *HBG1*, and *HBD*.¹³⁹ Three designed guide RNAs (gRNAs; G1, G2, and G3) can be transfected into

human cells to target various sections of the *HBB* gene; in 29 independent clones, specific editing of the *HBB* gene has been observed.¹³⁴ Genome editing in germline cells or early embryos can offer an opportunity for the treatment of genetic diseases. Recently, the naturally occurring C-C chemokine receptor type 5 (*CCR5* Δ 32) allele was successfully introduced into early human three pro-nuclei (3PN) embryos using the CRISPR-Cas system. However, the efficiency of HDR was low in the *CCR5* Δ 32 allele.¹⁴⁰ The *CCR5* Δ 32 gene was chosen because this gene encodes the major co-receptor used by the HIV-1 virus in targeting human immune cells.¹⁴¹ People with this allele show slow development of resistance to HIV infections.^{142,143} Subsequent procedures with premature human 3PN embryos generated 3PN embryos with the altered *CCR5* Δ 32 allele. Conversely, ZFNs have been used without a specific rat gene for the production of knockout rats.¹⁴⁴ In this process, the *GFP*, immunoglobulin M, and *RAB38* genes were targeted successfully to achieve the complete knockout of GFP, immunoglobulin M, and Rab38 transgenes without any cleavage at off-target sites.¹⁴⁵

Modifications of a genome, if introduced via its editing, can be heritable. Heritable alteration of the genome has been reported in rats after administering ZFN into one-celled embryos.¹⁴⁶ Several studies have generated multiple germline mutations in mice that turned out to aid in the development of genetically engineered animals. In mice, two loci, such as *TET1* and *TET2*, can be targeted through the modifications of CRISPR-Cas9. Treated parent mice have successfully transmitted this modification to the next generation when confirmed by DNA sequencing.^{147,148}

The genome-editing methods used in mammalian cells are not completely similar to those used in model organisms such as zebrafish. The Cas9/gRNA system has been successfully used in embryos to execute site-specific cleavage in zebrafish. The successful exogenous insertion of a foreign DNA fragment was then achieved as a donor DNA was provided during the experiment. DSBs induced by the Cas9 nuclease generate biallelic conversion of the *ETSRP* or *GATA5* gene in treated tissues. Microinjecting Cas9 mRNA and gRNA into zebrafish embryos leads to insertion of site-specific 1–24 bp or deletion of 7–32 bp. Cas9/gRNA has produced biallelic conversion of *ETSRP*, *GATA4*, and *GATA5* genes in targeted somatic cells.¹⁴⁹ In another study on zebrafish, the CRISPR-Cas system was used to target one hemizygous *EGFP* reporter gene and four endogenous loci (*TYR*, *GOL*, *MITFA*, and *DDX19*).¹⁵⁰ The resulting mutation rate of 75%–99% indicates that in most cells, the biallelic gene was disrupted. The five genomic loci in embryos were efficiently modified by the biallelic gene to show diverse phenotypes. CRISPR-Cas-induced mutations were highly specific in somatic tissues; additionally, germline transmission of these mutations has also been confirmed in the first generation.¹⁵⁰

Genome Editing in Stem Cells and iPSCs

Research on human induced pluripotent stem cells (iPSCs) and human embryonic stem cells (ESCs) has advanced the study of human genetics and cell-based therapies. Pluripotent stem cells can



self-renew and undergo unlimited division.¹⁵¹ These properties make them suitable for cloning and genotyping. Genome-modification tools have been successfully used to introduce highly specific alterations into iPSCs, rendering them distinguishable from background noise; this allows assessment of numerous developmental and disease-associated characteristics. Genome editing in human iPSCs aids in the production of synthetic organs and regenerative medicine, as well as in gene therapies.¹⁵² Therefore, interest in modified human iPSCs has increased because of their potential use in adapted cell therapy.^{151,153}

To study the gene functions in mice, researchers generally target ESCs via homologous recombination (HR); however, similar to HDR, HR was also unsuccessful in human ESCs.¹⁵⁴ ESCs differ between humans and mice, likely reflecting differences in DNA repair processes. Although both ZFNs and TALENs have been shown to be successful in editing iPSC genomes, cloning using CRISPR-Cas remains the most straightforward technique. Despite the successful achievement in targeting and modification of genes, isolation of edited iPSC clones was difficult and expensive. However, genome modification in treated human iPSCs is currently easily demonstrated by microscopy or flow cytometry. After modified iPSCs have been identified and genotyped, assessment should be made further for their pluripotency (extracellular and intracellular) and any chromosomal abnormalities.¹⁵³ Both human ESCs and iPSCs will likely play important roles in developing new technologies for modifications of the human genome.¹⁵¹ These technologies can also aid in engineering dendritic-cell-directed cancer vaccines, T cell immunotherapy, and experimental biology. Moreover, modifiable human ESCs and iPSCs can be used to create human cell lines for increased production of biomolecules, which have numerous industrial applications.^{155,156}

Human monogenic diseases (e.g., sickle cell anemia, spinal muscular atrophy, and X-linked adrenoleukodystrophy) can be easily treated by gene complementation or alteration. Recently, several studies on human monogenic diseases have successfully used genome-editing tools such as ZFNs, TALENs, and CRISPR-Cas9.^{155–159} Several gene therapy approaches have been developed and used for effective treatment of diseases such as X-linked severe combined immunodeficiency (X-SCID), Wiskott-Aldrich syndrome (WAS), and adenosine deaminase deficiency (ADA).¹⁵³ *Ex-vivo* gene complementation of retroviral-mediated gene therapy, which targets hematopoietic stem cells, has been used to permanently correct X-SCID-related abnormalities.¹⁶⁰ In an infection with the HIV-1 virus, CCR5 acts as a chemokine receptor that facilitates viral entrance into the host cell. Deletion of 32 bp at the coding region of this receptor renders it nonfunctional and consequently resistant to HIV-1 infectivity.¹⁴³

In other studies on HIV-1 infection in T lymphocytes or bone marrow hematopoietic stem cells, modification of the CCR5 receptor was achieved using ZFNs in CD4⁺ T cells.¹⁵⁷ This modification eventually activates NHEJ machinery, resulting in random mutations. Enforced expression of the four transcription factors, *OCT4*, *SOX2*, *KLF4*, and *c-MYC*, in patient-derived fibroblasts led to the develop-

ment of disease-specific human iPSC lines. Specific demarcation of such iPSCs enables the analysis of diseases such as Down syndrome, also called trisomy 21.¹⁵³ Modeling of Huntington's disease (HD) has also been performed using the CRISPR-based genome-editing technology and patient-derived iPSCs.¹⁵⁸ To create a Parkinson's disease (PD) model, ZFNs were used to introduce two-point mutations into a particular type of α -synuclein gene of human ESCs known as A53T. This is a dominant mutation in α -synuclein as commonly observed in a PD patient. As such, it is crucial evidence to acknowledge the sporadic form of the PD or less familial pattern of molecular pathogenesis.¹⁶¹ However, successful alteration of 1 bp restored the A53T mutation in patient-derived iPSCs without affecting the rest of the genome.¹⁶¹ Several similar genome-editing studies have been performed in iPSCs.¹⁵³ In one such study, a genome-editing tool was used to correct or disrupt a gene via point mutation.¹⁵³ These studies have also generated novel models for diseases such as Barth syndrome, HIV, and β -thalassemia.¹⁵³ Moreover, innovative research has been conducted with artificial nucleases. Most of the prior work has been aimed at increasing the efficiency of gene editing and developing a highly modified iPSC/ESC line.¹⁵³ The revolutionary research on this approach formed a TALEN-mediated Cas9-inducible human ESC line. Six different genes in this cell line were targeted using CRISPR, ultimately generating a double- or triple-knockout line.¹⁶² For therapeutic applications in patients with genetically linked diseases, genome editing is promising for the correction of disease-linked mutations in iPSC-derived progenitor cells. However, this approach is currently limited by the lack of well-established protocols.

Therapeutic Applications

The development of genome editing for therapeutic applications is challenging. Consistent alterations in multiple genes can have severe effects that induce complex polygenic disorders, which can be treated using advanced genome engineering tools such as ZFNs, TALENs, and CRISPR-Cas9. The therapeutic applications, possibilities, and challenges associated with programmable nucleases are illustrated in Figure 5.

Treatment of Hematological Disease

Inherited Hematological Disorders Gene therapy has been used for various human diseases, including hematological disease, cancer, AIDS, diabetes, heart failure, and numerous neurodegenerative diseases. More than 2,000 clinical trials using gene therapy have been carried out worldwide.¹⁶³ Several gene therapy products have been approved for marketing; these include Gendicine in China, Cerepro in Europe,¹⁶³ Luxterna,¹⁶⁴ and chimeric antigen receptor-modified T cells (CAR-T cells) in the United States.¹⁶⁵ Hematological diseases include genetic diseases and different types of malignancy. In order to develop a therapy for a genetic disease, it is necessary to identify a mechanism that can be used to overcome the effects of the causative mutation. The generation of iPSCs from patients with β -thalassemia has been performed using a non-viral method and a TALEN-based modification.¹⁶⁶ Modification of the mutation in these iPSCs can correct the function of the *HBB* gene. After sequencing, the *HBB* gene of targeted iPSCs did not show any additional mutations. This

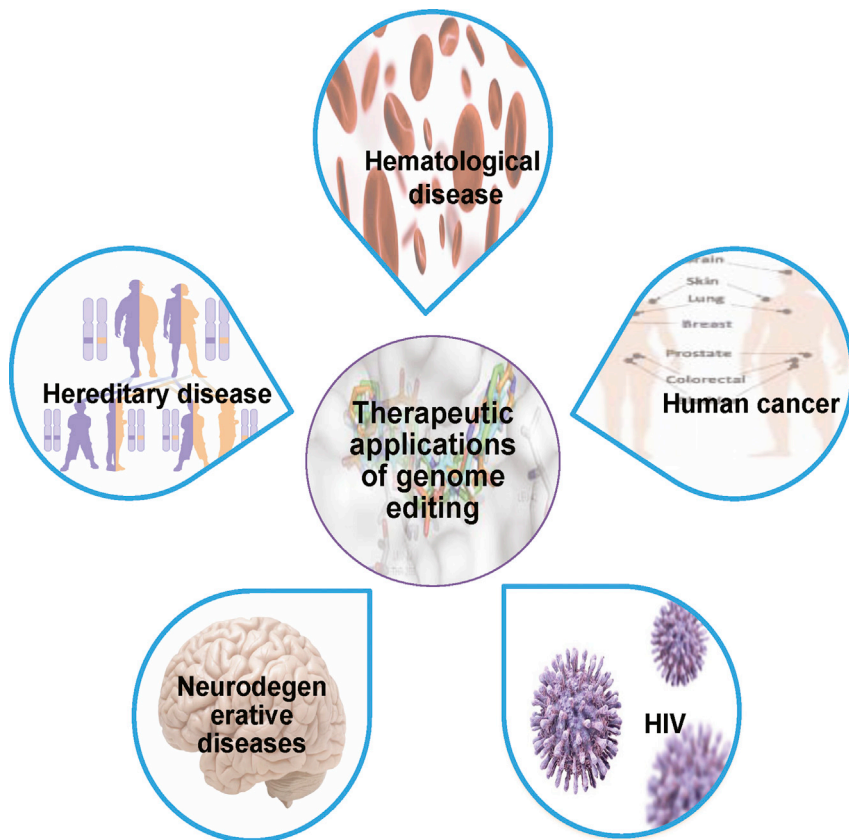


Figure 5. Schematic Diagram of Potential Therapeutic Applications of Genome Editing

approach can be used in the development of effective therapy against β -thalassemia.¹⁶⁶

Similarly, the genome-editing mechanism of CRISPR-Cas9 was also used to correct the β -thalassemia mutation.¹⁶⁷ In that study, the targeted cells showed a normal karyotype with proper pluripotency and no off-target mutations. The corrected human iPSC lines retain their expression of *HBB*, which suggests potential clinical application in gene therapy for β -thalassemia.¹⁶⁷ Sickle cell disease (SCD), another common genetic condition, is caused by a homozygous mutation in the sixth codon of the *HBB* gene. In this mutation, glutamic acid is replaced with a valine in the amino acid sequence. The consequence is abnormal production of β -globin and faulty red blood cells.¹⁶⁸ Recently, a ZFN-based strategy was used to repair two mutated β -globin alleles in iPSCs derived from a patient with SCD.¹⁶⁹ However, transcription of the repaired alleles was suppressed by the co-integration of a gene cassette in the first intron.¹⁶⁹ This issue can be resolved by using a *piggyback* transposon, which can facilitate the removal of this gene cassette in iPSCs without leaving unnecessary sequences.¹⁶⁸ Two TALENs were also designed to target the *HBB* gene mutation in SCD.¹⁷⁰ To correct the *HBB* gene, CRISPR-Cas9 technology was used in iPSCs derived from patients with SCD. This genome-editing technology relies on a donor template that includes the wild-type of *HBB* DNA, which shows normal activity.¹⁷¹

A common blood-related genetic disease, hemophilia A, is caused by several mutations in the blood coagulation factor VIII (*F8*) gene. Two types of chromosomal inversions that cover a fraction of the *F8* gene normally result in approximately 50% of hemophilia A.¹⁷² A particular TALEN pair is used to convert a 140-kbp chromosomal segment that passes through the *F8* gene in human iPSCs; this segment is responsible for hemophilia A. The modified segment can be returned to the original state using the same TALEN pair. The expression of *F8* mRNA was the same as that of wild-type in modified iPSCs cell lines. The results of this study indicated that a TALEN-based genome-editing mechanism can help correct gene rearrangements that cause genetic disorders such as hemophilia A.¹⁷³ Another genetic disorder, hemophilia B, is caused by the scarcity of blood coagulation factor IX, which is encoded by the *F9* gene. Mutations in this gene generally occur in exons 2–8.¹⁷⁴ Successful repair of the mutated *F9* gene was attained using ZFN technology *in vivo* combined with a targeting vector consisting of wild-type *F9* exons

2–8. The resulting gene targeting successfully repaired the hemophilia B phenotype. The repair mechanism was consistent, and neither failure nor restoration of mutation had been reported.¹⁷⁵

The phagocyte-NADPH oxidase enzyme complex includes a subunit called phagocyte oxidase (phox), which can produce reactive oxygen species (ROS). Mutations in phox cause the immunodeficiency chronic granulomatous disease (CGD).¹⁷⁶ The correction of X-linked CGD (X-CGD) in iPSCs was accomplished using ZFNs. In this case, ZFNs targeted a single copy of the therapeutic minigene *GP91^{phox}* located at the adeno-associated virus integration site 1 (*AAVS1*) locus. Further studies have shown that the inserted *AAVS1* alleles had no off-target insertions, and that these clones were converted to mature neutrophils with proper ROS production.¹⁷⁷ The heterozygous mutations in the *RUNX1* gene can lead to an unusual autosomal dominant disease called familial platelet disorder (FPD) and, further, to acute myeloid leukemia (AML). This gene encodes an important transcription factor that is involved in leukemogenesis. iPSCs of patients with FPD and/or AML having a *RUNX1* non-sense mutation Y260X showed deficient megakaryopoietic differentiation.¹⁷⁸ To correct the Y260X mutation, the donor vector was comprised of a cDNA sequence of exons 5–8 and two ZFNs specific for the *RUNX1* gene. This mechanism can correct FPD in the iPSC line to produce a wild-type variant of the gene and restored megakaryopoietic differentiation.¹⁷⁸



Fanconi's anemia (FA), a genetic disorder related to the failure of the bone marrow, causes hematological and solid malignancies.¹⁷⁹ FA is caused by a point mutation in the FA complementation group C (FANCC). This mutation can cause aberrant splicing that removes exon 4 from the FANCC gene.¹⁸⁰ An *in situ* study of this gene mutation was carried out using fibroblasts derived from FA patients.¹⁸¹ These authors applied CRISPR-Cas technology to amend the FANCC mutation for the intervention of the FA disorder. The results of this study were satisfactory, generating a high rate of corrected clones via HDR mechanism.¹⁸¹ Overall, the results of these studies indicate that genome-editing technologies can potentially alleviate inborn hematological disorders.

Acquired Hematological Disorders In acquired hematological disease, genome-editing tools can be used to examine gene function and to develop disease models. AML is a common myeloid disorder. AML can be treated using CRISPR-Cas9 to remove the *C4BPB* gene, which encodes the primary protein responsible for AML.²⁸ Similarly, ZFN genome-editing technology has been used to disrupt the Tet2 catalytic domain of the *TET2* gene, which is responsible for myelodysplastic syndrome in zebrafish.¹⁸² Conversely, myeloid malignant cells with multiple gene mutations can be modeled using the CRISPR-Cas9 genome-editing technology. This technology allows the production of animals with mutations in various genes. The majority of these mutations are found in cases of myeloid malignancies.¹⁷² Genome-editing technology together with transcriptomic analysis can be used as a novel approach to identify and confirm genes responsible for drug resistance in AML.¹⁸³ Genome-editing technology can be used in lymphoid malignancies as a new therapeutic approach.

Adult T cell leukemia (ATL) is enormously destructive to mature human T cells. A virus known as human T cell lymphotropic virus type 1 (HTLV1) is responsible for ATL.¹⁸⁴ Currently, there is no vaccine against HTLV1. A promising result was nonetheless obtained using ZFNs to target the HTLV1 provirus and destroy virally infected cells. The major advantage of this technology is the formation of two long terminal repeats (LTRs) for each provirus; LTRs possess enhanced binding affinity for their targets.¹⁸⁵ ZFN technology has proven to be a promising tool to combat ATL. Burkitt's lymphoma (BL) is a latent viral infectious disease. The causal organism of BL is a herpesvirus known as the Epstein-Barr virus (EBV). Recently, the CRISPR-Cas9 system was used to disrupt the EBV genome in a BL cell line. The results of that study indicated that CRISPR-Cas9 suppressed the activity of EBV and restored the pathway of cellular apoptosis.¹⁸⁶ BL cell lines require MCL-1, an anti-apoptotic protein, for their functioning and survival. The deletion of this protein by CRISPR-Cas9 technology induces apoptosis in the BL cell lines.¹⁸⁷ Genome-editing technologies can be used to achieve a better understanding of the molecular mechanisms involved in inherited and acquired hematological disorders. However, in using these technologies, we need to optimize gene delivery and avoid destructive off-target side effects.

Treatment of Hereditary Disease. Targeting a specific sequence is challenging when correcting defective genes in patients with inherited

disorders. Currently, gene therapy and RNAi have shown the greatest precision in targeting specific sequences.¹⁸⁸ This ability currently renders gene therapy and RNAi as the two most powerful therapeutic tools for the treatment of hereditary diseases. Gene therapy involves the reinstallation of omitted gene function via viral transgene expression. Optimized oligonucleotide designs showed a localized distribution with improved safety profiles. RNAi thus enabled the targeting of numerous genes in one tissue simultaneously. RNAi suppressed the expression of faulty genes by knocking down target mRNA. Gene therapy and RNAi are thus currently used to treat severe combined immunodeficiency (SCID), transthyretin-related hereditary amyloidosis, WAS, cancer, and age-related macular degeneration.^{189–195} However, these technologies still have limited efficacy in treating certain genetic diseases.¹⁸⁸ To overcome these limitations, several nuclease-based genome-editing approaches have been developed and used for the potential treatment of hereditary diseases. Naturally occurring mutations can confer resistance in non-monogenic diseases such as cardiovascular disease, Alzheimer's disease (AD), and hemoglobinopathies.³⁴

Genome-editing technologies are efficient tools for introducing protective mutations into affected individuals. NHEJ with programmable nucleases was successfully used to inactivate the *HTT* gene in HD and the *FGFR3* gene in achondroplasia. In both cases, natural allele integrity was maintained. Cell-culture models, currently used to study HD and AD, and to develop suitable therapies against neurodegeneration, are discussed later in this review. NHEJ-based technology was also successfully used to treat nucleotide expansion disorders such as spinocerebellar and Friedreich's ataxia.¹⁸⁸ HDR has also been used to convert a mutant sequence to wild-type sequence in CCR5 in HIV and PCSK9 in hypercholesterolemia.^{196,197} Mouse models are used to assess the efficacy of CRISPR-Cas9-mediated *in-vivo* genome editing in adult animals. Tyrosinemia type I (HTI) is a lethal genetic disease caused by a mutation in the enzyme fumarylacetoacetate hydroxylase (FAH).³⁴ A point mutation of G to A in the last nucleotide of exon 8, which leads to the exclusion of exon 8 during splicing, causes the abnormality in mice and humans. The instability of the FAH protein causes the accumulation of toxic metabolites, resulting in severe liver damage.¹⁹⁸ To correct the G-to-A splicing mutation of Fah protein in the mouse model, single-strand DNA (ssDNA) was used along with unguided Cas9 technology for the treatment of hereditary tyrosinemia.³⁴ Another example is primate DMD. DMD is caused by three different mutations in the dystrophin gene. These mutations result in the loss of dystrophin and degeneration of primate muscle, similar to the phenotype of DMD patients.¹⁹⁹ CRISPR-Cas9 was delivered directly into the muscle cells of mice with DMD using adeno-associated viral delivery. Once delivered, CRISPR-Cas9 excised the mutated exon, generating an excised dystrophin. DMD mice that underwent this treatment showed better muscle strength without off-target effects.^{200–202} In summary, the CRISPR-Cas9 system can be used as an efficient tool to correct or mitigate genetic diseases.

Treatment of Cancer. Cancer is a polygenic disease caused by defects in multiple genes associated with oncogenes and tumor suppressor

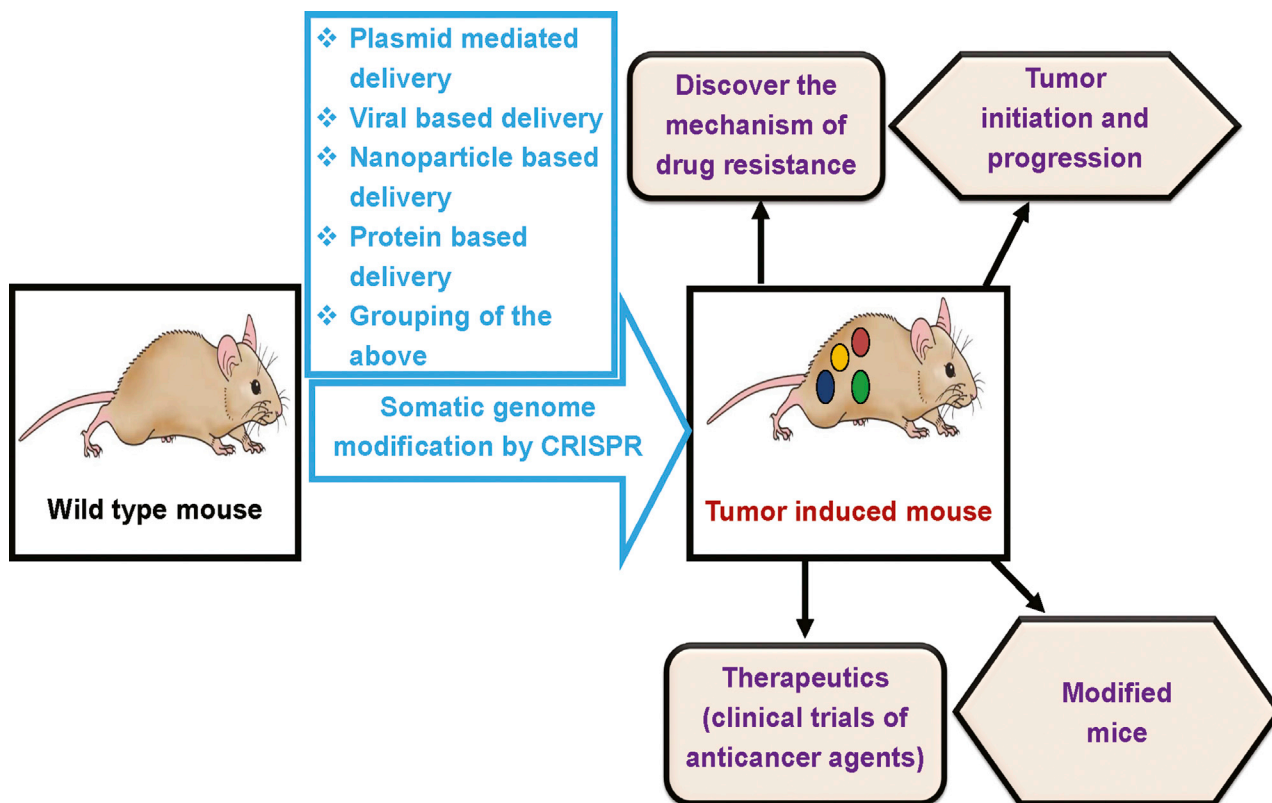


Figure 6. A Schematic Diagram for Production of Genetically Engineered Mouse Models Using Programmable Nuclease (CRISPR-Cas9)

Genetically engineered mouse models (GEMMs) can be used to discover mechanisms of drug resistance and those of tumor initiation and progression, and to develop new anticancer drugs.

genes. The genomes of normal cells and cancer cells are important for modeling cancer and uncovering genes that are responsible for the overall process. Genetically engineered mouse models (GEMMs), with specific mutations in genes, oncogenes, and tumor suppressors, are used to study cancer biology.²⁰³ A phenomenon called passenger mutations, occurring directly or indirectly, aids in the conversion of normal cells into cancer cells by activating oncogenes or inactivating tumor suppressor genes. CDNA-based overexpression and RNAi-based inactivation can cause off-target effects. However, CRISPR-Cas9 produces less off-target integration and, therefore, is frequently used in cancer research. Different types of cell lines with one or more targeted mutations are suitable for exploring the effects of mutations associated with cancer phenotypes.

CRISPR-Cas9 technology also enables analysis of oncogenic signaling pathways via sequential or multiplex gene editing.²⁰³ Using GEMMs and non-germline GEMMs, researchers have uncovered the fundamental features of tumor instigation, maintenance, and evolution. Moreover, these models can be used to screen anticancer agents and identify drug-resistance mechanisms (Figure 6).^{204,205} Genome-editing technology based on CRISPR allows us to generate large stockpiles of ESC lines with numerous arrangements of basic or restrictive mutations in oncogenes and tumor suppressor genes.

The main advantage of the CRISPR-Cas9 system in mouse cancer models is the systematic generation of models with multiple oncogenic alleles. This property aids in assessing allele-specific tumor development and therapeutic responses. The CRISPR-Cas9 method is also used for improving existing cancer models. These next-generation models will help us to better understand and discover functional remedies against cancer.²⁰³

In recent years, this system has also been used to edit the somatic genome both *ex vivo* and *in vivo*. *Ex-vivo* editing of the *TP53* tumor suppressor gene has been performed in *Eμ-Myc* transgenic mice.²⁰⁶ A similar approach was used for *ex-vivo* disruption of the mixed heredity leukemia 3 tumor suppressor gene in AML.²⁰⁷ In another study, CRISPR-Cas9 was used to rapidly generate mouse models of AML by *ex-vivo* modification of single or multiple genes via lentivirus.²⁰⁸ These studies showed that CRISPR-Cas9 *ex-vivo* somatic genome editing can be used to rapidly produce mouse models of several human malignancies. Similarly, CRISPR-Cas9 was used in hepatocytes of living animals to deliver the plasmids of Cas9 and sgRNAs, respectively, targeting the *PTEN* and *TP53* tumor suppressor genes *in vivo*.¹⁰⁹ These studies show that somatic modification of cancer genes by CRISPR technology in wild-type mice can proficiently onset certain types of cancer.



For curative modification of single or multiple mutations, CRISPR-based technology offers efficient delivery and editing via viral or non-viral methods. CRISPR-Cas9 technology can be used to modify T cells and the immune response.²⁰⁹ Additionally, this technology may be used for specific *ex-vivo* manufacturing of immune cells for potential immunotherapy. For example, new CAR-T cells possess an introduced chimeric antigen receptor.^{210,211} In summary, the use of CRISPR-Cas9 technology in cancer biology has allowed us to shift from fundamental research to experimental and translational applications.

Several viral infections are involved in carcinogenesis; these include: (1) hepatitis B and C viruses in liver cancer, (2) EBV in nasopharyngeal carcinoma, and (3) human papillomavirus (HPV) in cervical cancer. Inactivation of these oncogenic viruses may alleviate tumorigenesis.¹⁶³ The CRISPR-Cas9 genome-editing system can be used to alleviate such carcinogenesis via defense against oncogenic viral infections. Antiviral and anti-proliferative effects were obtained using the CRISPR-Cas9 system in HPV-mediated cervical carcinoma and EBV-mediated BL cells.^{186,212} In both cases, treatment with CRISPR-Cas9 inhibited proliferation of tumor cells while reducing the overall viral load.

If cancer is treated as a genetic disease, CRISPR-Cas9 can be used as a genome-editing tool to correct oncogenic abnormalities in the genome. Collaboration between genetic mutations and epigenetic alterations may cause the initiation and progression of cancer. CRISPR-Cas9 can be used to correct genetic mutations in monogenic diseases and transform the epigenetic states of a cell.¹⁶³ With the help of sgRNAs, dead Cas9 (dCas9; a Cas9 should lose endonuclease activity due to point mutations at the endonuclease domains) and epigenetic modifiers can enter target sites for epigenetic regulation; this is a prospective tool in anticancer therapeutics. Recently, there has been increased interest in improving anticancer immune responses, and resistance to chemotoxicity and radiotoxicity. Cancer is polygenic and heterogeneous in nature; this presents the main obstacle in CRISPR-Cas9-mediated cancer therapy.¹⁶³

Bladder cancer is a common urologic cancer that can be mitigated by traditional chemotherapy and radiation treatment. However, the limitations of these traditional treatments include: (1) mass cell killing, (2) nonspecific targeting, (3) serious side effects, and (4) numerous abnormalities of a genome.²¹³ Recently, the CRISPR-Cas9 system was used in combination with modular AND gate circuits, human cancer-specific promoter of the telomerase reverse transcriptase gene, and a human bladder-specific promoter gene. This circuit can identify bladder cancer cells and is used to effectively treat bladder cancer.²¹⁴ The CRISPR-Cas9 genome-editing system can be used to produce logic circuits. The Cas9 protein of the CRISPR-Cas9 system can be joined with sgRNA to generate an effector complex that leads to a double-strand DNA cleft.²¹⁵ These circuits were shown to robustly and precisely inhibit the proliferation of carcinoma cells in the bladder; these circuits also restored apoptosis in these cells while lowering their motility.²¹⁶ Chromosomal relocation plays a vital role

in the expression of genes that show therapeutic activity against cancer.

In human non-small-cell lung cancer (NSCLC), the combination of two oncogenes (echinoderm microtubule-associated protein-like 4 [*EML4*] and anaplastic lymphoma kinase [*ALK*]) was shown to play an important role.²¹⁶ Using CRISPR-Cas9 technology with a viral delivery method in somatic cells, researchers successfully rearranged the chromosomes of adult mammals *in vivo*. This process produced a mouse model of *EML4-ALK*-mediated lung cancer.²¹⁷ These mouse models provide information about the molecular mechanisms underlying tumor formation; this can be used to assess drug resistance and the efficacy of targeted therapeutics *in vivo*. Conversely, CRISPR-Cas9-mediated technology for chromosomal rearrangement is limited by spaces between the excised sites and their affinity for Cas9.²¹⁸ Colorectal cancer (CRC), another commonly occurring human carcinoma, is caused by several mutations in normal colon epithelium. The loss of *APC* gene and successive mutations in *KRAS* (encoding Kirsten rat sarcoma viral oncogene homolog), *SMAD4* (encoding the SMAD protein), and *TP53* (encoding the p53 protein) genes are mainly responsible for colorectal carcinogenesis. In a rodent model, mutations in these genes were found to induce intestinal adenocarcinoma similar to human CRC.²¹⁹ Using CRISPR-Cas9 genome-editing methods, genetic alterations can be introduced into human intestinal cells.^{82,215} sgRNAs and CRISPR-Cas9 can be used to target the *APC*, *SMAD4*, and *TP53* tumor repressor genes. This technology has also been used to isolate single cells from human organoids and to generate mutations that recapitulate the adenoma-carcinoma sequence.²¹⁹

Treatment of Neurodegenerative Diseases. The most common neurodegenerative diseases are mainly differentiated as either age-reliant or selective neurodegeneration. The pathogenesis of common neurodegenerative diseases, such as PD, AD, HD, amyotrophic lateral sclerosis (ALS), and spinal muscular atrophy (SMA), remains unclear.²²⁰ To date, there are no effective therapies for these diseases. However, the generation of cellular models will help acquire information about the pathogenesis of these conditions and will lead to facilitated drug screening. Using stem cells for cell replacement was recently explored as a possible therapy for these diseases. The prevalence of various neurodegenerative diseases is higher in developed countries such as the United States (around 7 million occurrences).²²¹ As mentioned earlier, genome-editing tools are used to prevent the inheritance of AD. In future applications, it may be possible to use these tools to develop curative therapies against neurodegeneration. Genome-editing technologies, such as TALENs, ZFNs, and CRISPR-Cas9, have already been used to alter or generate genetic mutations and model particular neurodegenerative diseases (Figure 7).^{222,223} In higher animals, it is difficult to produce large-animal disease models, necessitating the use of cell models. Additionally, the lack of ESC lines, derived from large animals, is a major obstacle in gene manipulation.²²⁰

Recent progress in genome-editing technologies has enabled the production of large-animal models for exploring neurodegenerative

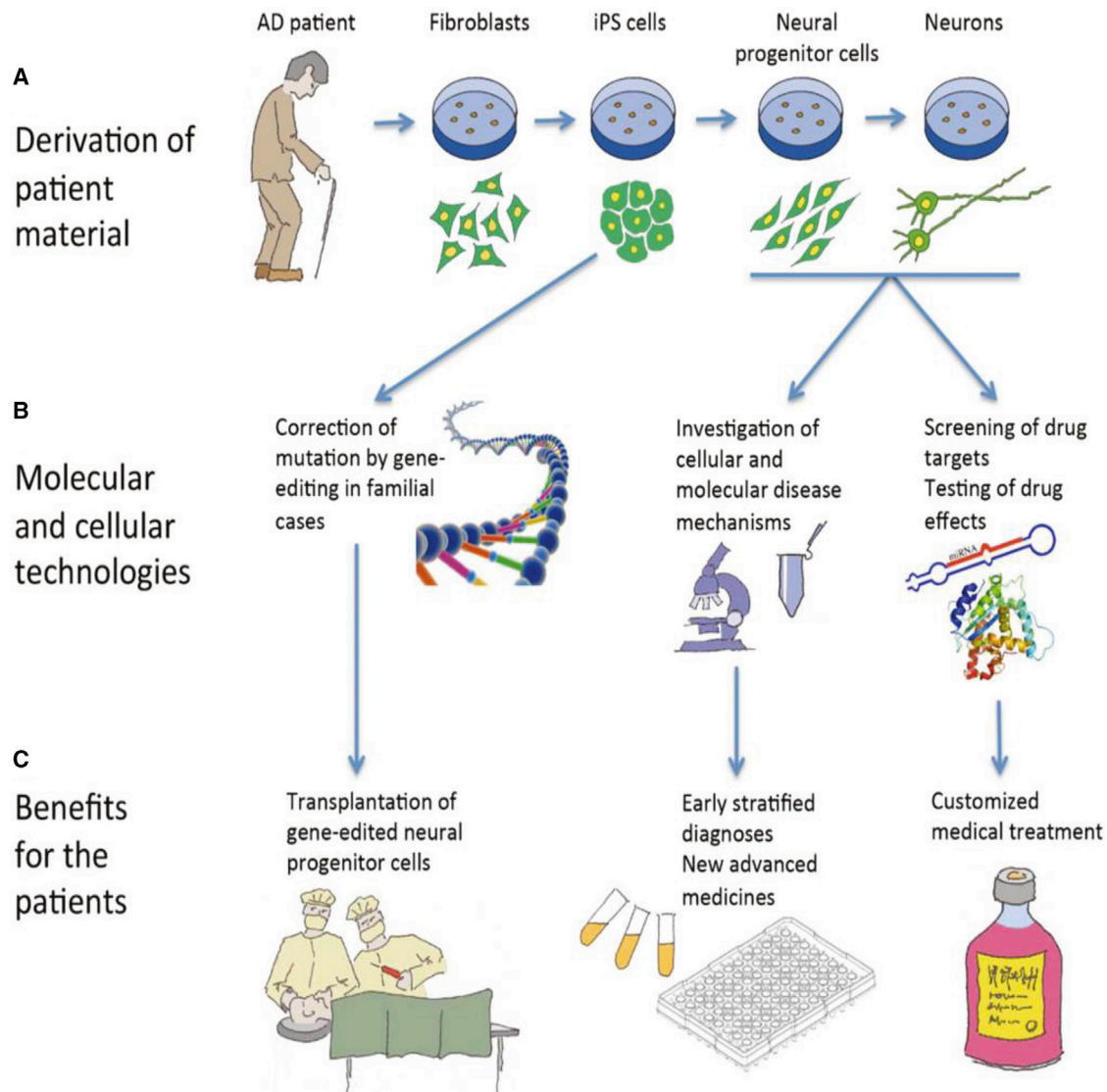


Figure 7. Schematic Illustration of the Use of Induced Pluripotent Stem Cells in Relation to Alzheimer's Disease

(A) Induced pluripotent stem cells (iPSCs), derived from a skin biopsy acquired from a patient with Alzheimer's disease (AD), are differentiated into neural progenitor cells and neurons. (B) In familial cases, the disease-causing mutation can be corrected by gene editing of the iPSCs; the neural progenitor cells and neurons can be used for research and drug screening. (C) Patients can benefit from cell therapy, better diagnostic procedures, customized treatments, and novel medical approaches. Reproduced from Freude et al.,²²³ copyright (2014) Creative Commons Attribution 4.0 International.

diseases.²²⁰ The CRISPR-Cas9 system can be used to create mutations that impede the open reading frame and inactivate the gene. Hence neurodegenerative diseases, caused by malfunction of specific genes in animals, can be easily studied using CRISPR-Cas9.²²⁰ CRISPR-Cas9 genome-editing technology can also be used to assess mosaic mutations in various types of cells. CRISPR-Cas9 can target each gene in an embryo, which is advantageous in generating animal models of neurodegenerative diseases. This technique is particularly useful in large-animal models. Neurodegenerative diseases, such as PD, can originate from mutations in the *PARKIN* and *PINK1* genes.

In animal models, CRISPR-Cas9 can be used for targeted silencing of candidate genes.²²⁰ This may enable us to mimic mutations observed in patients with PD. Neurodegenerative diseases can also originate from mutant cytotoxic proteins. PD and HD are partially caused by mutations in α -synuclein and polyglutamine extended huntingtin, respectively.²²⁰ Using CRISPR-Cas9 technology in mammalian cells can efficiently generate these proteins, while other tools, such as NHEJ, can be used to increase their production.²²⁴ Promising results in human immunotherapy are also being reported. Human ALS is frequently caused by the *C9ORF72* gene, which encodes dipeptide repeat (DPR) proteins. CRISPR-Cas9-based knockout screening of



the human genome was thus used to identify suppressors and enhancers of *C9ORF72*-encoded DPRs. Using this technology, numerous modifiers were identified; one of these modifiers, *TMX2*, ameliorated endoplasmic reticular stress in a patient with *C9ORF72* ALS, resulting in dramatic survival of neurons.²²⁵

Treatment of HIV. The first case of HIV infection was reported in 1981.²²⁶ Since then, HIV has become a major public health concern, affecting more than 35 million people globally.²²⁶ Although antiretroviral therapy (ART) can reduce the symptoms of HIV-1, it is not possible to achieve full recovery. The main obstacle for curative therapy is perseverance of HIV reservoirs, which cannot be eliminated by antiretroviral therapy.²²⁷ HIV viral DNA assimilates into host genomes, forming organized viral reservoirs. It is possible that deleting or neutralizing viral DNA would eliminate HIV persistence.²²⁸ Some limitations of current antiviral therapies include drug toxicity, resistance to antiretroviral therapy, and failure to eliminate a dormant viral infection. In addition, the medications and expenses of lifelong treatment, as well as large numbers of patients with HIV/AIDS, render treatment grueling and costly. To eliminate dormant viral reservoirs, it is important to focus on individualized therapies that can block viral DNA and show minimal drug toxicity.²²⁹

Nuclease-mediated genome editing is an encouraging approach in therapeutic applications against HIV-1.^{229,230} The engineered nucleases mainly include ZFNs, TALENs, and CRISPR-Cas9; these can disrupt HIV-1 proviral DNA that has converted into active DNA and integrated into the host genome. These technologies can also interrupt the entry of HIV-1, which occurs via co-receptors C-C chemokine receptor 5 (CCR5) or C-C-C chemokine receptor 4 (CXCR4). CCR5 can be targeted by ZFNs; this treatment for patients with HIV-1 is in phase II medical trials.^{229,230} Because the entry of HIV-1 requires co-receptors, such as CD4 and either CCR5 or CXCR4, these co-receptors are potential targets for therapeutic applications. A deletion of 32 bp in the *CCR5* gene of a patient with HIV-1 eliminated the existing infection and rendered the patient resistant to infections with R5 type HIV-1.^{143,231}

As discussed previously, antiretroviral therapy cannot eliminate HIV from dormant reservoirs. Dormant viral reservoirs are mostly found within memory CD4⁺ T cells and can persist for approximately 60 years after receiving antiretroviral therapy.²³² The cleavage of HIV-1 proviral DNA, induced via CRISPR-Cas9, is dose dependent and does not show adverse effects in patients. The use of ZFNs has also been successful in eliminating the HIV-1 provirus by targeting the transactivation response element of HIV-1 LTR. However, using ZFNs to eliminate proviral DNA needs to be further optimized by protein engineering.¹⁵⁵ The delivery of ZFNs into human CD4⁺ T and CD34⁺ hematopoietic stem progenitor cells (HSPCs) to target the *CCR5* gene was assessed using a mouse model of HIV infection.

Using an adenoviral vector in patients with HIV showed promising results. This vector expresses a ZFN and was used to target the *CCR5* gene in CD4⁺ T cells isolated from 12 patients with HIV.¹⁰⁴

The CRISPR-Cas9 gene-editing system can also remove proviral DNA by a sgRNA-guided method.²³³ This technology of proviral DNA removal by CRISPR-Cas9 also helps to inactivate viral genes in other diseases. CRISPR-Cas9-mediated removal of HIV proviral DNA was achieved using sgRNAs, which can target preserved sites within the U3 region of viral LTR in dormant infections.²³⁰ In addition to inactivation of proviral DNA and prevention of replication in infected cells, CRISPR-Cas9 can provide resistance against recurrent HIV infections and can target multiple spots inside the HIV genome.²³⁴ The Cas9 protein of CRISPR-Cas9 can persist in human HSPCs. HSPCs can then differentiate into monocytes and macrophages without undesirable side effects. TALENs and CRISPR-Cas9 can also be used for natural deletion of *CCR5Δ32* in iPSCs with a piggy-Bac transposon donor sequence.²³⁵

An HIV-GFP Jurkat cell line (JLat10.6) has also been used to test the efficacy of the CRISPR-Cas9 system in silencing HIV-1 DNA.²³⁶ The results of that study indicated that CRISPR-Cas9 effectively targeted and silenced HIV-1 proviral DNA in the JLat10.6 cell line. This spurred the notion to match gRNA with a targeted viral DNA sequence.²³⁶ Recently, internal antisense long non-coding RNAs (lncRNAs) were tested as novel tools for inhibiting the HIV-1 provirus, which was expressed from the *NEF* gene.²²⁹ High expression of *NEF* lncRNA can cause viral inhibition, whereas low expression shows the opposite result. The 5' LTR region may be a suitable target for the CRISPR-Cas9 genome-editing system.²²⁹ Several antiviral therapies can be used to control viral infections. However, the virus is untraceable in the blood, which poses a major problem. Recent studies show that the CRISPR-Cas9 genome-editing system can act as a novel antiviral tool by targeting viral coding regions. The system can be used to block assimilation and progression of HIV-1 infection, to eliminate hidden viral reservoirs, and to confer lasting immunization against HIV-1.

Application of Genome Editing in Plants Crop Improvement

Traditional approaches to improving crop species have generally depended on conventional and transgenic breeding methods. However, these methods suffer from several limitations. The former is limited by a declining genetic base that depends on germplasm collections. In addition, randomly occurring variants are generated by mutagenic effects. The latter generates genetically modified (GM) crops, which is concerning with respect to health and environmental safety. The addition of foreign genes into a genome can limit commercialization and will possibly require a complex regulatory system. To overcome these difficulties, newly introduced techniques based on site-specific nucleases (SSNs) may be suitable tools for successful genome editing.

Genome modification in plants was first conducted in the early 1990s; however, due to the low efficiency of genome engineering, this research did not advance. In recent years, precise genome modification in model plants and other important crops has been revolutionized with the help of SSNs.^{237,238} To date, research on SSNs has identified three common programmable nucleases: ZFNs, TALENs, and

**Table 1. Practical Applications of Nucleases in Crop Plants**

Crop Plants	Treated Tissue	Used Nucleases	References	Practical Applications
Rice	embryo	TALENs	250	resistance to bacterial blight
			251	
			283	
		Cas9/sgRNA	284	
			285	
			286	
			287	
Maize	maize cells	TALEN and CRISPR-Cas9	288	reduction of phytate (inositol phosphate), creation of male sterile plants
			289	
		meganucleases	290	
			291	
		ZFN	242	
Soya bean	hairy root and somatic embryo	ZFN	292	production of seeds with high monosaturated oleic acid and low polyunsaturated fatty acid, linoleic acid
		Cas9/sgRNA	293	
		TALENs	294	
Cotton	embryogenic callus cells	meganucleases	295	resistance to herbicide
Canola	immature seed	ZFN	296	decreased levels of palmitic acid and increased total levels of C18 fatty acids
Wheat	genomic DNA	CRISPR-Cas9	285	resistance to powdery mildew
		TALEN	248	
		sgRNAs	297	
		Cas9/sgRNA	248	
Sorghum	immature embryos	CRISPR-Cas9	284	expression of clover fluorescence protein
Barley	grains of winter barley	TALENs	298	creation of homozygous mutants
			299	

CRISPR-Cas-mediated RNA-guided DNA endonucleases, which are used for editing plant genomes (Table 1).³ Among these, the RNA-based CRISPR-Cas system is the most suitable for use in various organisms, especially in plants.²³⁹ The overall editing mechanism depends on the introduction of DSBs at target sites commonly repaired by either HR or NHEJ process.²⁴⁰ NHEJ can generate gene knockouts by producing frameshift mutations in coding sequences; alternatively, HR results in gene substitution, combination, or accumulation. To date, most genome modification procedures have mainly focused on model plant species. As such, enormous efforts have been invested into crop plant research. In the tobacco plant, resistance against one or more herbicides was introduced by a missense mutation in donor templates, which was then inserted into protoplasts along with ZFNs. Similarly, the same *ALS* gene in a tobacco protoplast was replaced using TALEN-mediated genome modification.²⁴¹

Continuous expression of ZFN and a simple donor molecule add the *PAT* herbicide tolerance gene at the *IPK1* locus. This genome modification imparts herbicide resistance and leads to high accumulation of phytate and low levels of inorganic phosphate caused by *IPK1* expression.²⁴² GM organism (GMO) crops have been commercially used in the recent past. However, these crops are still not accepted

in the developed countries because of serious health and environmental considerations. Conversely, specific manipulation of genomes by SSN can be used to overcome problems associated with classical transgenic breeding and can prevent the introduction of foreign genes and proteins, reducing the probable risks associated with transgenic procedures.^{243,244} The US Department of Agriculture (USDA; Washington, DC, USA) has provided public authorization to use ZFN techniques for production of GM corn.²⁴⁵ The regulatory agenda for GM crops in European Union (EU) countries is mainly focused on genome modification procedures rather than on final products. Therefore, crops modified by genome-editing tools, such as CRISPR-Cas9 and others, may not be classified as GMOs.^{244,246} Finally, site-specific techniques can offer more accurate methods for crop improvement and may be promising biotechnological tools for plant breeding.

Trait Improvement

Without the domestication of plants, we could not maintain the balance between supply and demand or provide food, medicine, chemicals, renewable materials, animal feed, and biofuels. The process of domestication includes improvement of crop performance and crop properties that are directly related to human welfare. Genome editing



can accelerate the overall plant breeding process by inserting or modifying genes that can directly improve the characteristics of crops.

Among genome-editing methods, CRISPR-Cas9 is more advantageous because multiple traits can be customized simultaneously using this procedure. The simplest form of gene modification is NHEJ-mediated gene knockout. This technique was successfully used to eliminate genes that negatively affected food quality, decreased resistance to pathogens or diseases, and decreased the value of end products.²⁴⁷ TALEN and CRISPR-Cas9 can be used to target genes located at the mildew resistance locus (*MLO*) in the wheat plant. The *MLO* homoalleles can be effectively knocked out to promote resistance to powdery mildew in the wheat plant.²⁴⁸ SSNs enable the addition of numerous genes near existing transgenic loci to facilitate stacking of targeted molecular traits. This mechanism can introduce multiple traits into crop plants with minimal risk of segregation, which is the main obstacle in classical plant breeding and in modern genetic engineering methods.

After stacking, the complete group of transgenes can be moved to another plant because such a group acts as a solitary locus. A maize line, containing an herbicide resistance marker and an artificial ZFN target site, was produced using this process.²⁴⁹ CRISPR-Cas9 techniques are accessible and straightforward to use in both forward and reverse genetics, aiding in basic research conducted with model plant species. This is helpful for generating genomic data, and for more rapid gene discovery and trait improvement in various plant species.²⁴⁷ A modification TALEN technique was developed by engineering *HAX3* from the Brassicaceae pathogen *Xanthomonas campestris*. These techniques have been widely used in many crop plants including rice, barley, and maize. The genes that are responsible for disease vulnerability in rice can be easily mutated by TALEN to produce disease-resistant rice with normal phenotypes.²⁵⁰ In rice, numerous mutants have been produced using TALENs to knock out eight *Brachypodium* genes with high efficiency.²⁵¹

Genome-editing methods, such as CRISPR-Cas9, play a vital role in identifying novel traits in common profitable crop plants. The delivery and expression of engineered nucleases in plant cells are critical because some plant species do not show positive results. For generating novel traits, it is important to establish a balance between the specific plant tissue and the method used for transformation; this is a major issue that needs to be addressed. To overcome these difficulties, Gemini virus-mediated replicons can be used to transmit DNA for genome engineering in various plant species.²⁵² New approaches to plant breeding, along with in-depth understanding of the whole-plant genome, will facilitate the development of important traits in plants.

Minimizing Off-Target Mutagenesis Risks of Nucleases

Genome-editing nucleases are the multipurpose tools that are extensively applied for studying the potential of genetic materials, producing GMOs, and preclinical investigation of genetic diseases. However, the frequency of off-targeted activities (more than 50%) is a major

concern in *RGEN* target sites, particularly in therapeutic and clinical applications.²⁵³ To minimize the off-target mutations, a specific proofreading approach needs to be elucidated. However, CRISPR-Cas9 is reported as an editing approach with proofreading capability. The mechanism of CRISPR-Cas9 was first discovered in bacteria, where it is used as an immune response against invading pathogens such as bacteriophages.^{254–256} In the early stages of this discovery, the CRISPR-Cas9 system was mainly used as a tool for genome editing in various types of cell lines. Later, living organisms, such as *C. elegans*,^{255,257} zebrafish,¹⁴⁹ mice,^{258,259} rats,^{147,148} rabbits,²⁶⁰ and monkeys,²⁶¹ were more easily modified with CRISPR-Cas9 than with TALENs or ZFNs. In humans, the CRISPR-Cas9 system can also play an important role in correcting genetic mutations such as those involved in β -thalassemia,³² cystic fibrosis,²⁶² hemophilia A,³³ cataracts,²⁶³ hereditary HTI,³⁴ and DMD.³⁵

Numerous studies have shown that in human cells, nucleases are involved in host defense mechanisms to fight against viruses such as hepatitis B^{264,265} and HIV.^{233–236} Frequent use of nucleases has raised concerns about nonspecific activity at off-target sites, especially when nucleases are used for clinical purposes. Nonspecific activity at off-target sites may cause stable and injurious cytotoxicity or even tumorigenicity, impeding normal cellular function in humans. Nonspecific activity at off-target sequences is less frequent in the case of CRISPR-Cas9 than in cases of ZFNs and TALENs.^{50,266–269} Off-target cleavage activity of CRISPR-Cas9 can be decreased by mutagenesis-related approaches or by eradicating the nuclease function of CRISPR-Cas9. For deactivation of the RuvC nuclease domain, an aspartate-to-alanine mutation is highly effective and attenuates the activity of CRISPR-Cas9.²⁵⁴ In this procedure, just one strand is cleaved off dsDNA, producing a simple gap rather than an entire blunt dsDNA break, which is less efficient in promoting HR.

Several groups have shown that the double-nick or DSBs strategy increases cleavage at on-target sites; this can be used in combination with wild-type (WT) Cas9, which effectively reduces off-target mutations.^{270–272} This nicking activity may cause point mutations; hence this process is also risky.²⁷³ The fusion of FokI nuclease with an inactive Cas9 protein can control the genome-editing process. In the gene-editing process, both *Streptococcus pyogenes* Cas9 (SpCas9) and *Staphylococcus aureus* (Sa) Cas9 have been used extensively. However, the specificity and cleavage activity of SaCas9 is higher than that of SpCas9; this is because the former can be guided to a 21- to 23-nt site, whereas the latter can only recognize a 17-nt sequence.^{274,275}

Combining the Cas9 protein and sgRNA-mediated on- and off-target cleavage has been used for DNA plasmid transfections in different cell lines. This process results in overexpression of the Cas9 protein and sgRNA, which may cause off-target mutagenesis because larger amounts of DNA are transfected.²⁹ As the number of interactions increases, the chances of off-target site cleavage by Cas9 binding also increase. Direct delivery of Cas9 into cells has been used to reduce the off-target effects. The Cas9 protein is unstable and has a moderately



short half-life, making its presence transient. Using the Cas9 protein instead of the Cas9 plasmid produces 13 times more on-target cleavage compared with off-target cleavage.²⁷⁶ In a 72-hr time-course experiment, the Cas9 protein was no longer detected after 24 hr, whereas a noticeable increase in Cas9 plasmid transfection persisted for up to 72 hr.²⁷⁶ Chemical modification at the 3' end of sgRNAs improves their cleavage activity and specificity during genome editing.²⁷⁷

Future Prospects of Genome Editing

As we have shown, genome-editing technologies have the potential to significantly advance cell-based therapy and improve our understanding of many genetic diseases. Efforts made over the last decade are leading to the generation of specific and permanent multiple-hit pluripotent cell (PC) lines for reprogramming into disease-linked cell types. However, well-developed protocols for these methods are still lacking. It is important to optimize this methodology and to achieve competent gene alteration and biallelic gene editing before translating these approaches into clinical applications. In a few studies, the relative effectiveness of gene editing was compared among the available options such as ZFNs, TALENs, and CRISPR/Cas9. Recently, it was shown in human pluripotent stem cells (hPSCs) that the CRISPR-Cas9 system is more effective than TALEN-mediated gene editing.⁸³

In some cases, a combination of techniques is preferable to using traditional approaches to improve efficiency; examples of such combinations are integrase-defective lentiviral vectors (IDLVs)/ZFN,^{278,279} transposon/CRISPR,³² and TALEN/CRISPR (iCRISPR).¹⁶² For patient therapy, genome-editing approaches must precisely target disease-associated mutations in either iPSC-derived progenitor cells or in mature cells for autologous transplant; these procedures must be considered safe and not disturb other loci in the genome. Interestingly, a complete genome analysis by Veres et al.²⁸⁰ confirmed that a slight risk of off-target mutations is posed by both CRISPR-Cas9 and TALEN targeted to the sortilin 1 (*SORT1*) gene in human ESCs (hESCs). Presently, there are promising advances in gene alteration for a variety of diseases. Schwank et al.²⁶² recently corrected the function of cystic fibrosis transmembrane conductance regulator (*CFTR*) gene using CRISPR-Cas9 in adult stem cells (SCs) derived from patients with cystic fibrosis (Figure 8). In that study, treatment with CRISPR-Cas9 generated few off-target effects. The results of the forskolin-response assay, performed in that study, confirmed that the activity of disease-linked transmembrane receptor was recovered. However, cystic fibrosis affects multiple organs (Figure 8),²⁶² making it difficult to treat cystic fibrosis using the CRISPR-Cas9 system.

Recently, Sánchez-Rivera and Jacks²⁰³ predicted that nuclease-based genome editing will be a vital link, connecting the bench and clinical applications in cancer treatment. Successful expansion of genome profiling for extensive characterization of patient-derived tumors is creating comprehensive roadmaps for developing cell- and whole-animal-based empirical approaches. Single or multiplex

nuclease- and/or small-molecule-based technologies will advance the development of personalized genome editing, which can be used to rapidly recognize genotype-specific weaknesses and harmful synthetic interactions.²⁸¹ Such personalized techniques can potentially enable rapid identification of lethal mechanisms and development of respective therapeutic approaches.²⁸²

Several technical restrictions limit the usage of CRISPR-Cas9 for therapeutic targeting of oncogenes in human patients, and prospects of this gene therapy are currently controversial. Recently, a study used this technology to achieve accurate *in-vivo* genetic alterations in the hepatic tissue of adult mice. This study successfully corrected an inherited genetic disorder via HDR in a mouse model.³⁴ Future developments in this technology will permit therapeutic modifications of single or various driver mutations. Such developments will also increase the effectiveness of editing and delivery via CRISPR-Cas9 using both non-viral and viral delivery vectors. In addition to modifying cancer-linked mutations, CRISPR-Cas9 components can be employed for specific *ex-vivo* editing of immune cells for immunotherapeutic applications. For example, CRISPR-Cas9 components can be used to improve T cells modified with the chimeric antigen receptor (CAR-T cells);²¹⁰ in these cells, CAR is incorporated into a safe harbor locus.

Currently, CRISPR-Cas9 is a leading RNA-programmed gene-editing platform. The field of genome alteration has undergone a rapid scientific revolution that will transform basic biological and biomedical research.²⁵⁴ Genome editing can be used in various types of iPSC-based therapy and cancer therapy, offering exciting prospects for better assessment of disease progression and development of effective treatments.

Concluding Remarks

There is tremendous promise in using the CRISPR-Cas9 system for targeted editing of the genome in cells and whole organisms. This technique will advance human therapies, agricultural biotechnology, and microbial gene engineering. The revolutionary discovery of the CRISPR-Cas9 genome-editing system will impact the fields of modified medicine, human genetic alteration, and drug development. Nonetheless, various obstacles to genome-editing technology still need to be overcome. The major concerns associated with genome editing in higher organisms are off-target mutations in the genome and precision in inserting Cas9 into a cellular system. Overcoming these limitations will advance the field of human gene therapy. Both off-target mutations and imprecise delivery of Cas9 into the cell can be lethal because they can cause cellular alterations and death. Several studies have shown that Cas9-guided RNA complexes can alleviate genetic disease when injected into an adult mouse model. Traditional methods, such as nucleic acid and protein delivery, can be helpful in identifying suitable delivery methods for genome-editing nucleases. In addition to nucleases, supplementary methods for genome editing have been considered. Recently, another technology, called triplex-forming peptide nucleic acids, was successfully used for genome modifications. These technologies

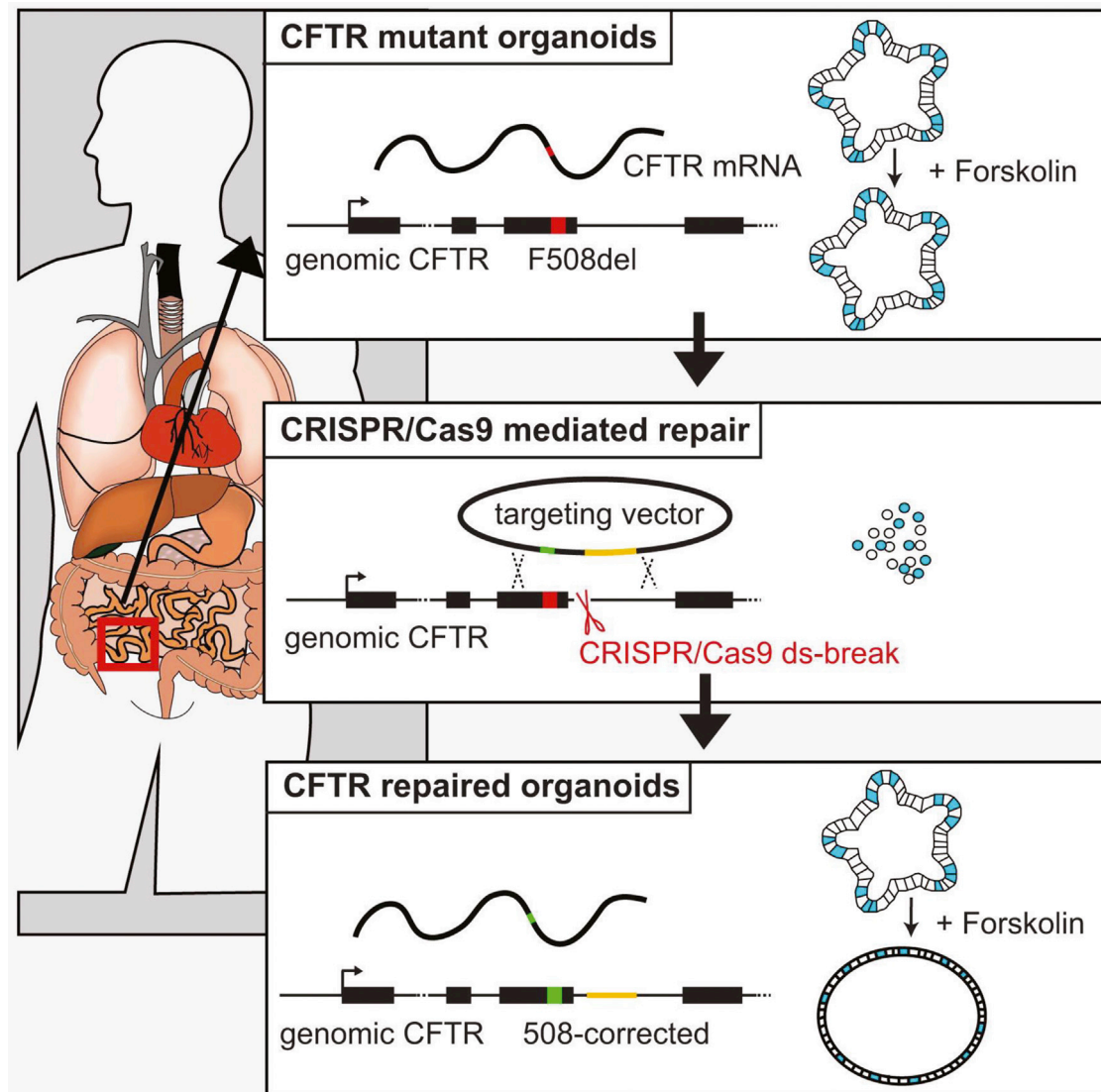


Figure 8. Schematic Illustration Showing Functional Repair of *CFTR* by CRISPR-Cas9 in Intestinal Stem Cell Organoids Acquired from Patients with Cystic Fibrosis

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will revolutionize the fields of medical and pharmaceutical sciences by helping us identify biochemical pathways in infections and by simplifying drug delivery methods. The limiting factors of genome editing in animal and plant species need to be addressed before application in humans because of the potential impact on human welfare. Thus, detailed molecular studies and suitable disease models are required before using genome editing in the treatment of human diseases.

AUTHOR CONTRIBUTIONS

S.K.S., M.S.R., and K.-H.K. designed the study; F.K.S. and S.M.K.R. drafted the manuscript; S.K.S. extracted all figures; M.A.H.M.J. and S.M.R.I. partially wrote the manuscript; S.K.S., M.S.R., and K.-H.K.

wrote and edited the manuscript. All authors reviewed and approved the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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