Therapeutic Genome Editing and In Vivo Delivery

by

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(Under the Direction of Dexi Liu)

ABSTRACT

Improvements in the understanding of human genetics and disease have led to an increased interest in therapeutic genome editing using engineered nucleases. Various approaches have been taken in the past to develop an effective and safe system for sequence specific editing. Compared to earlier nucleases such as ZFN and TALEN, the low cost and ease of producing CRISPR/Cas9 systems has improved the feasibility of therapeutic genome editing. CRISPR/Cas9 genome editing has shown great potential to correct genetic mutations implicated in monogenic diseases and to eradicate viral infections in preclinical studies. Several CRISPR/Cas9-based therapeutics have reached clinical testing, including treatments for inherited red blood cell disorders and Leber Congenital Amaurosis 10, as well as edited T cell-based cancer therapies. Further advances in therapeutic genome editing will require safer and more efficient in vivo CRISPR/Cas9 delivery systems and optimization of HDR efficiency.

INDEX WORDS: Genome editing, therapeutic genome editing, gene therapy, CRISPR/Cas9, TALEN, ZFN, genetic disease

THERAPEUTIC GENOME EDITING AND IN VIVO DELIVERY

by

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1. INTRODUCTION

The completion of the Human Genome Project in the early 2000s has given rise to the identification of over 21,000 protein-coding genes (1). These genes are differentially expressed or silenced in different cells, generating various complex cell structures and functions. While many diseases occur due to multiple genetic and environmental factors, approximately 5,000-8,000 diseases originate from a single genetic mutation (2). Many of these monogenic diseases are symptomatically manageable with small drug molecules, exogenous proteins or genes, or antisense oligonucleotides, but these drugs often have undesirable off-target effects. Therapeutic genes and antisense oligonucleotides targeting the genetic mechanism of the disease often require repeated dosing for long-term efficacy. The next step in optimizing disease treatment and prevention may be to develop a genome editing-based therapy capable of permanently correcting the causative mutation. The major goal of this review is to provide a summary of the mechanisms of genome editing, the reasons for the emerging interest in CRISPR/Cas9 compared to other engineered nucleases, the current progress in developing CRISPR/Cas9 delivery systems, and the current preclinical and clinical applications of CRISPR/Cas9 genome editing.

2. PRINCIPLES OF GENOME EDITING

Genome editing requires an engineered nuclease containing a sequence-specific DNA-binding domain and a nuclease domain that cleaves both DNA strands. The resulting double-stranded break induces cellular DNA repair via a non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathway. NHEJ is an error-prone process that generates a small insertion or deletion mutation, disrupting the reading frame and function of the target gene. In contrast, HDR is an error-free process that incorporates a homologous sequence at the cleavage sites where the nuclease cleaves both the 5' and 3' ends of the coding sequence. HDR enables insertion of an exogenous gene at the target site to replace a mutant gene with a healthy copy. These two DNA repair pathways compete with one another. NHEJ is generally favored because the process can occur at any stage of the cell cycle. In contrast, HDR primarily takes place during the G2 and S phases of the cell cycle when a homologous DNA sequence is present in the nucleus (3,4). The cell-cycle dependency of HDR limits its applications.

3. TYPES OF ENGINEERED NUCLEASES FOR GENOME EDITING

Introduction

Well-studied engineered nucleases include zinc finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats associated protein 9 (CRISPR/Cas9). Table 1 summarizes these genome editing systems.

Table 1. Unique Features of the Most Commonly Studied Engineered Nucleases[a]

Nuclease	Mechanism	Target Site	Advantages	Disadvantages	Ref.
ZFN	α-helix of zinc finger domain mediates DNA binding; Fok-1 nuclease cuts one DNA strand.	9-18 bp per monomer, 18-36 bp per pair; G-rich sites	Small size allows packaging into many types of viral vectors; limited off-target effects	Need linker sequences to fuse ZF domains together; targeting limited to G-rich sites $(GNN)_N$	(5-7)
TALEN	RDV of the TALE domain allows binding to a specific DNA sequence; Fok-1 nuclease cuts one DNA strand.	14-20 bp per monomer, 28-40 bp per pair	Limited off-target effects	Difficult to engineer due to large number of TALE repeats; large size makes TALEN difficult to package for delivery	(6,8)
CRISPR/ Cas9	gRNA mediates DNA binding, and Cas9 induces a double strand break at the site	17-20 bp, PAM sequence	Easier and less expensive to engineer; Cas9 can be reused with different sgRNAs; suitable for multiplexing	Susceptible to off-target effects; requires PAM sequence	(6,9)

[a] Fok-1: forkhead box transcription factor 1; gRNA: guide RNA; RVD: repeat variable di-residue; PAM: protospacer adjacent motif; sgRNA: small guide RNA; TALE: transcription activator-like effector

Zinc Finger Nucleases (ZFNs)

ZFNs are constructed by fusing several zinc finger domains and 1 Fok-1 nuclease domain. Zinc finger domains contain two β sheets coordinated with a zinc ion and an α helix that recognizes 3 bp of DNA (5). Typically, 3 individual zinc finger domains are chemically linked to form an array that binds a total of 9 bp (Figure 1). Zinc finger libraries have been built and can be used to find zinc finger proteins that recognize specific DNA sequences. The Fok-1 nuclease domain cleaves one strand of DNA, and therefore, 2 ZFN constructs are needed to make a double-stranded DNA break (6).

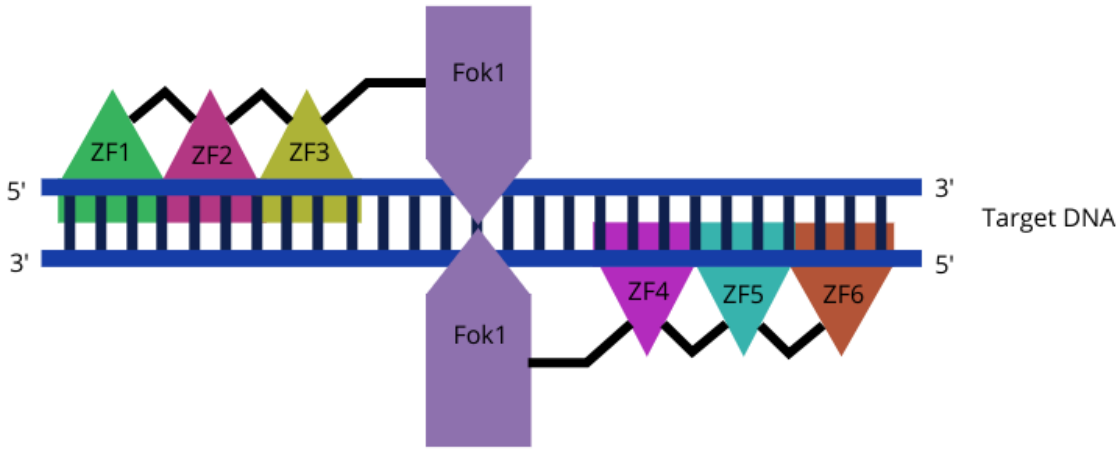

Figure 1. ZFN Target Recognition

Transcription Activator-Like Effector Nucleases (TALENs)

TALENs contain a transcription activator-like effector (TALE) domain that comprises a series of repeated 33-34 amino acid sequences (Figure 2). The 12th and 13th amino acid residues of each TALE repeat form a repeat variable diresidue (RVD) that determines which nucleotide the monomer recognizes. Constructing a targeted DNA-binding domain requires chemical linkage of 14-20 TALE repeats with RVDs modified to recognize each nucleotide in the

target sequence (8). As with ZFNs, Fok-1 serves as the nuclease, cleaving one DNA strand, and a pair of TALEN constructs is required to create a double stranded DNA break.

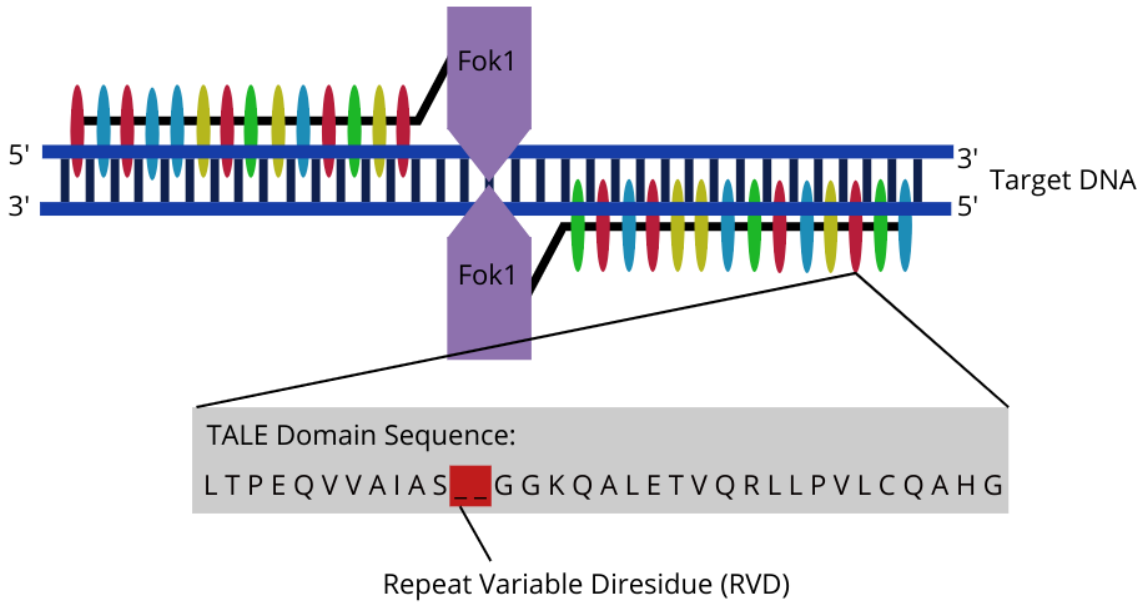

Figure 2, TALEN Target Recognition

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9

CRISPR systems function naturally in bacteria, providing adaptive immunity against phage infection. When a phage injects its DNA into the bacterial cell, the bacterial genome integrates a “spacer” DNA sequence from the phage into the CRISPR locus. Repeat sequences in the CRISPR locus separate the spacers incorporated from different phages. On subsequent exposure to the phage, the bacterial cell transcribes the spacer into a CRISPR RNA (crRNA) that guides a Cas nuclease to the complementary phage DNA sequence. The Cas nuclease recognizes a protospacer-adjacent motif (PAM) sequence specific to the phage genome, enabling targeted phage DNA cleavage and simultaneous preservation of the spacer within the CRISPR locus (10). Class I CRISPR systems contain multiple subunits, whereas Class II systems comprise a single subunit (Table 2). Among Class II CRISPR systems, Cas9 and

Cas12a recognize DNA sequences, whereas Cas13a targets RNA. Cas12a forms staggered-ended dsDNA breaks, whereas Cas9 produces a blunt cut, making Cas9 more applicable to targeted genome editing (9,11,12).

Table 2. Major Types of CRISPR/Cas Systems[a]

CRISPR Class	Subtype	Function	Ref.
Class I (Multiple Subunit)	Type I: Multiple Cas Proteins (Cas3, Cas4, Cas5, and Cas6)	crRNP formed by crRNA and multiple Cas proteins recognizes the PAM sequence and base pairs with the DNA, forming an R-loop; individual Cas is recruited to the crRNP and cleaves the DNA using its helicase and nuclease domains.	(13)
	Type III: Cas10, Cmr3, Csm3, Csm6, Csx1	crRNP is only active when the DNA is being transcribed to RNA; crRNA recognizes the target RNA; Cmr3 or Csm3 endoribonuclease cleaves the RNA at 6 nt intervals; Cas10 cleaves the ssDNA target and generates cyclic oligoadenylate signaling molecules that stimulate Csm6/Csx1 to cleave target RNA.	(14)
Class II (Single Subunit)	Type II: Cas9	crRNP formed by Cas9 and the crRNA/tracrRNA; crRNP recognizes the PAM sequence and base pairs with a DNA sequence of ~20 nt; Cas9 makes a blunt-ended double-stranded break a few bases upstream of the PAM, via two nuclease active sites	(9)
	Type V: Cas12a	crRNP formed by Cas12 and crRNA recognizes the PAM sequence and base pairs with a DNA sequence of ~20 nt; Cas12 makes staggered-ended dsDNA breaks, via one nuclease active site	(11)
	Type VI: Cas13a	crRNP formed by Cas13 and crRNA base pairs with an RNA sequence of ~20 nt; Cas13 cleaves the target RNA	(12)

[a] Cas: CRISPR associated protein; crRNA: CRISPR RNA; crRNP: CRISPR RNA nuclear protein; tracrRNA: trans-activating CRISPR RNA; PAM: protospacer-adjacent motif; ssDNA: single stranded DNA

CRISPR/Cas9 systems consist of a Cas9 nuclease and a guide RNA (gRNA) (Figure 3). The 17-20 nucleotide CRISPR RNA (crRNA) component of the gRNA binds to the target DNA sequence by complementary base pairing. The trans-activating CRISPR RNA (tracrRNA)

component activates the Cas9 nuclease by binding to the REC I domain. The PAM interacting domain of Cas9 mediates DNA binding, and the bridge helix domain initiates DNA cleavage. The HNH and RuvC nuclease domains cut one DNA strand each, resulting in a double-stranded break. The Cas9 nuclease is most commonly derived from *Streptococcus pyogenes* and recognizes the PAM sequence 5'-NGG-3' (9).

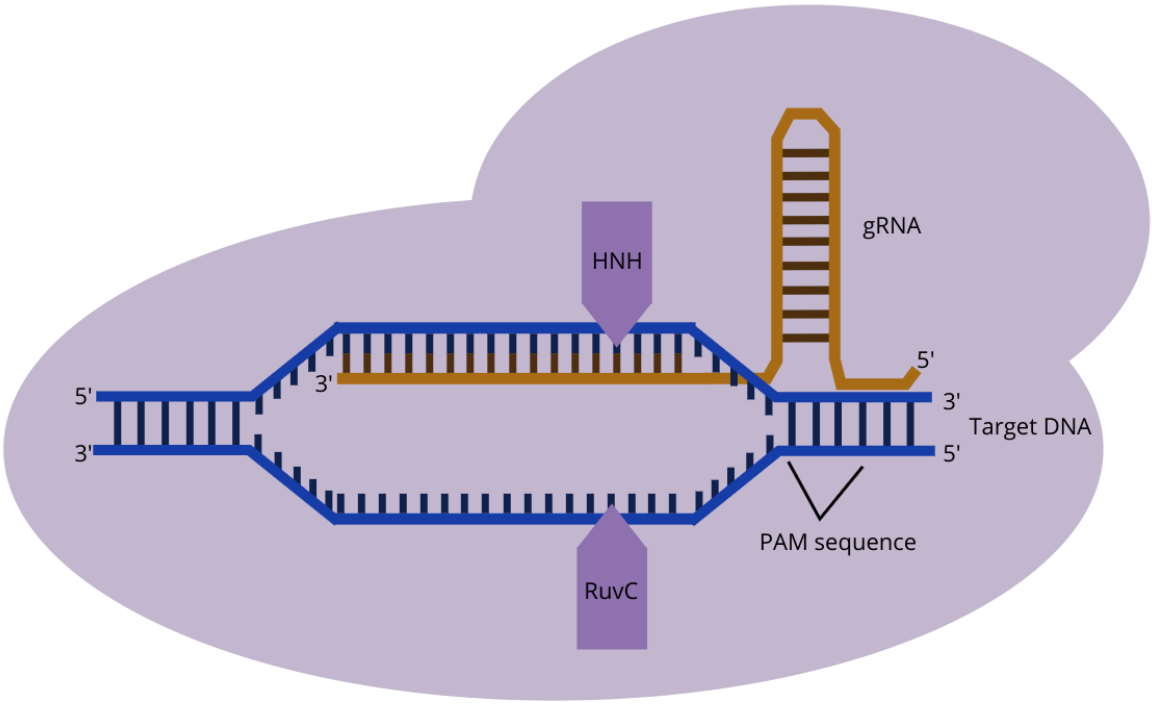

Figure 3. CRISPR/Cas9 Target Recognition

Although ZFN and TALEN systems produce targeted genome edits, sequence-specific DNA binding protein domains are difficult and expensive to engineer. In contrast, CRISPR-based genome editing systems target the genome via an easily synthesizable RNA molecule. Additionally, the Cas9 nuclease is reusable with different gRNAs, enabling multiplexed gene targeting. CRISPR/Cas9 is more prone to cause off-target effects than the

other 2 nucleases (6), but the advantages of this system outweigh this limitation. Therefore, genome editing studies focus primarily on CRISPR/Cas9.

4. CRISPR/CAS9 DELIVERY SYSTEMS

CRISPR/Cas9 systems are readily deliverable via a plasmid encoding Cas9 and the gRNA. Such plasmids are stable, simple to produce, and easy to deliver *ex vivo* with physical methods or *in vivo* with viral vectors. However, long-term Cas9 expression may result in off-target DNA cleavage or host immune responses (15,16). Delivering a Cas9 mRNA can limit the duration of Cas9 activity but may also reduce delivery efficiency due to RNA degradation in the bloodstream. Therefore, RNA is less commonly used (17). An increasingly popular strategy has been to deliver a Cas9-gRNA ribonucleoprotein (RNP) complex. The Cas9 protein protects the gRNA from degradation, and the pre-formed RNP complex can rapidly produce the desired edit (18). However, *in vivo* delivery requires the engineering of a safe and efficient nonviral carrier to accommodate the large RNP.

Physical Delivery

Physical CRISPR/Cas9 delivery is primarily suitable for germline editing in animals and *ex vivo* gene editing (19-22). The two most common physical methods are microinjection and electroporation. Microinjection employs a micropipette to inject essential editing materials precisely into the nucleus, pronucleus, or cytoplasm of a single cell (23). This method requires great skill and is less efficient than electroporation, which applies an electric field to transiently increase membrane permeability, allowing for the passage of materials into the cytoplasm (24). Microfluidic devices recently developed show potential for optimizing the throughput of electroporation and enable the scaled-up manufacture of gene-edited cell therapies. For example, Lissandrello et. al. (2020) designed a microfluidic continuous-flow electrotransfection device in which a solution containing the T cells and mRNA flows through the device, and electrodes in contact with the stream produce electric fields to transfect the cells. At the optimal electric field strength, pulse length, and pulse number, the device achieved up to 95%

transfection efficiency without significantly reducing cell viability. At a cell concentration of 50 million cells/mL, the device maintained 72-75% transfection efficiency and was able to process approximately 20 million cells per minute. With 500 million cells using an optimal experimental condition, 75% transfection efficiency and 91% cell viability were achieved (25).

Viral Delivery

AAV vectors are the most common viral vectors used in CRISPR/Cas9 delivery due to their relatively low immunogenicity and lack of transgene integration into the host genome. Additionally, different AAV serotypes exhibit different tissue tropisms, enabling cell-specific delivery. However, the SpCas9 gene has a size of 4.1 kb, and adding the gRNA and necessary regulatory elements causes the size to exceed the 4.7 kb packaging capacity of the AAV vector (26). This limitation necessitates the use of either a dual AAV vector that carries the SpCas9 gene and the gRNA separately or a smaller Cas9 variant such as *Staphylococcus aureus* Cas9 (SaCas9), which spares approximately 1 kb of space. One weakness of this system is that such variants often have stricter PAM sequence requirements that limit target flexibility compared to SpCas9 (27).

Adenoviral and lentiviral vectors have more forgiving packaging capacities than AAV. However, safety concerns necessitate further modification of these vectors for *in vivo* use. For example, the well-documented occurrence of significant immune responses has limited the clinical use of first- and second-generation of adenoviral (ADV) vectors (28). High-capacity adenoviral vectors (HCAdVs) lacking viral genes have reduced immunogenicity and expanded packaging space (29). HCAdVs have efficiently delivered CRISPR/Cas9 systems targeting the covalently closed circular DNA (cccDNA) of the hepatitis B virus (HBV) and oncogenes in the human papillomavirus (HPV) genome *in vitro* (30,31). Ongoing and future research will reveal *in vivo* safety and genome editing efficacy of HCAdVs.

Lentiviral vectors have a packaging capacity of approximately 8 kb (32). Pseudotyping with foreign viral surface proteins facilitates cellular uptake and confers tissue-specific tropism

(33). However, because the transgene integrates into the host genome (34), the use of lentiviral vectors is limited primarily to *in vitro* CRISPR/Cas9 screening for gene function (35). Novel integrase-deficient lentiviral vectors (IDLVs) circumvent this limitation by expressing the transgene episomally. Ortinski et al. demonstrated the successful use of IDLVs *in vivo* CRISPR/Cas9 genome editing (36), but therapeutic applications of IDLVs carrying CRISPR/Cas9 remain unexplored. Table 3 summarizes each viral vector type.

Table 3. Summary of the Viral Vectors Used in CRISPR/Cas9 Delivery

Vector	Advantages	Limitations	CRISPR/Cas9 Applications	Ref.
Adeno Associated Virus (AAV)	Low immunogenicity; Low host genome integration; Tissue-specific serotypes	Low packaging capacity (4.7 kb)	Can be used therapeutically *in vivo*	(26,37,38)
Adenoviral Vector (AdV)	Low host genome integration; High-capacity and lacking viral genes (HC-AdVs); higher packaging capacity (~36kb)	Significant host immune response with traditional AdV	Has been used experimentally *in vivo*, not to the same extent as AAV	(28-31,39)
Lentiviral Vector	8 kb packaging size; Low immunogenicity; Pseudotyping with foreign surface proteins	High host genome integration → increased off-target effects of Cas9	Primarily used for CRISPR/Cas9 screens; Integrase-deficient lentiviral vectors have potential for *in vivo* applications	(32-36,40)

Nonviral Delivery

Synthetic nonviral carriers can be designed with the appropriate carrying capacity to package the CRISPR/Cas9 DNA, RNA, or RNP. However, multiple barriers to *in vivo* delivery make it a challenge to engineer an efficient nonviral delivery system. When administered systemically, the carrier must protect the material from degradation by proteases and nucleases. Ideally, the carrier should not adsorb plasma proteins, as this can result in the formation of a protein corona that is recognized and degraded by the immune system. The tight junctions between the endothelial cells of the blood vessels limit the efficiency of extravasation, and the density and negative charge of the extracellular matrix interfere with interstitial transport. Delivery efficiency may also be limited by off-target cell uptake, low cell membrane permeability, or degradation in the endosome (41). These limitations must be considered when designing a nonviral carrier.

Nonviral CRISPR/Cas9 delivery systems developed so far employ cationic materials to protect the CRISPR/Cas9 components from degradation. Nanocarriers formulated with cationic lipids or polymers are considered suitable for *in vitro* CRISPR/Cas9 delivery because the positive charges on nanocarriers form complexes with editing materials via electrostatic interactions, facilitating efficient encapsulation (42). However, it has been reported that the cationic lipid- and polymer-based formulations impart significant cytotoxicity (43,44). Several chemical modification strategies have been explored to reduce the cytotoxicity of cationic polymers. For example, cyclohexanedicarboxylic anhydride (CCA) or folic acid (FA) were used to substitute the NH_2 groups of the PEI (45). Alternatively, adding glutathione during PEI nanoparticle synthesis reduces cytotoxicity (46). Studies have also proposed wrapping PEI with a macrocyclic compound such as cucurbituril or cyclodextrin (47,48). Conjugating the PEI nanoparticle with polyethylene glycol (PEG) also helps modulate the surface charge and prevents degradation of the nanoparticle in the bloodstream (49). Studies have similarly suggested the benefits of conjugating cationic lipids with an anionic material such as hyaluronan

(50,51). Novel cationic lipid-assisted nanoparticles contain lipids such as BHEM-Chol and neutral block copolymers such as PEG-b-PLGA in a ratio that optimizes the surface charge for efficient CRISPR/Cas9 encapsulation and reduces cytotoxicity. In 2018, Liu et al. successfully delivered CLANs *in vivo* to knockout the neutrophil elastase (NE) gene in a type 2 diabetes mouse model, reporting a CLAN uptake efficiency of 94.6% in neutrophils and indel frequencies of 19.5% and 27.3% in the eWAT and liver, respectively (52). In another 2018 study, they used CLANs to deliver a macrophage-specific promoter-driven Cas9 plasmid to knockout the netrin-1 (Ntn1) gene in type 2 diabetes mice, reporting an indel frequency of 23.2% in monocytes and macrophages isolated from the adipose tissue (53). In 2020, they used CLANs to co-deliver a Cas9 plasmid with three gRNAs and a type 1 diabetes (T1D) autoantigen peptide, effectively programming dendritic cells to activate T1D antigen-specific Treg cell proliferation *in vitro* and *in vivo* (54).

Biodegradable materials are considered as less cytotoxic alternatives to cationic lipids and polymers. For example, zeolitic imidazolate frameworks (ZIFs) are able to encapsulate the CRISPR/Cas9 RNP and rapidly decompose in the endosome (55). Wang et al. (2020) designed ZIFs specific to mouse retinal pigment epithelium (RPE) tissue by complexing the ZIFs with silica and further modifying them with PEG and the RPE-targeting ligand all-*trans* retinoic acid. These modified ZIFs exhibited no significant cytotoxicity with an injection dose of up to 200 μg/ml and did not adversely affect RPE cell function when injected subretinally in mice. The RNP delivered by the ZIFs induced gain-of-function mutations in the tdTomato transgene, significantly increasing the area of RPE tissue expressing the fluorescent signal (56).

Biodegradable cationic polymers have also demonstrated potential as safer alternatives to PEI. For example, Zhang et al. (2020) found that *in vivo* delivery of a Cas9/gRNA plasmid with paclitaxel via a chitosan nanocomplex disrupted VEGFR2 with 33.4% efficiency in tumor tissue and inhibited tumor growth by 70%. *In vitro*, the nanocomplex exhibited significant cytotoxicity against cancer cell lines such as HepG2 and H22, but not against non-cancerous

HEK293T cells (57). In another study, Gao et al. (2020) found that polyamidoamine (PAMAM)-poly (β-amino ester) (PBAE) copolymer nanoparticle-mediated delivery of CRISPR/Cas9 plasmids targeting HPV oncogenes inhibited cervical cancer growth at a concentration of 50 μg/mL and was not significantly cytotoxic at doses below 75 μg/mL. In contrast, the commercial cationic lipid transfection reagent HP exhibited cytotoxicity at a dose of 1 μL/mL, which is lower than the standard HP dose of 2 μL/mL. The PBAE nanoparticles also showed no significant *in vivo* toxicity, and were able to prevent tumor progression in HPV-infected mice (58). Bioreducible cationic lipidoids containing cleavable disulfide bonds have also been explored. Cationic lipidoid nanoparticles have successfully delivered Cas9 mRNA to human mesenchymal stem cells, knocking out the neuron restrictive silencing factor (NRSF) gene and inducing the differentiation of hMSCs into neuron-like cells. The differentiation was evaluated by the increased expression of synaptophysin brain-derived neurotrophic factor, neuron-specific enolase, and neuron-specific growth-associated protein (59).

Other materials in nonviral formulations have been explored to load the CRISPR/Cas9 system, enhance cellular uptake, or enable tissue-specific delivery. In CRISPR-Gold systems, a gold core is conjugated with thiolated DNA, which forms a complex with the Cas9/gRNA RNP, the donor DNA template, and a cationic material. Gold nanoparticles functionalized with the cationic copolymer PAsp(DET) have been used to delete and replace the mutant *DMD* gene in a mouse model of Duchenne Muscular Dystrophy (60). Gold nanocarriers functionalized with cationic cell-penetrating peptides such as polyarginine and HIV-1 TAT have also been developed. Ray et al. (2018) used polyarginine-functionalized gold nanoparticles to knock out SIRP-α gene in macrophages and enhance cancer cell phagocytosis (61). Wang et al. modified gold nanoclusters with the Cas9/gRNA RNP and the HIV-1 TAT peptide, then further encapsulated the materials in an outer lipid shell. This system knocked out Polo-like kinase 1 (*Plk1*) in mouse tumor tissue, leading to a 70% decrease in *Plk1* expression and 75% inhibition of melanoma progression (62). Zhang et al. (2018) further modified the outer lipid shell with

galactose to target the mouse liver and knock out proprotein convertase subtilisin/kexin type 9 (*Pcsk9*). This system inhibited *Pcsk9* expression by 60% and decreased plasma LDL cholesterol by about 30% (63).

Other materials were explored to control the release of CRISPR/Cas9 under specific conditions, allowing for local delivery with minimal off-target uptake. For example, gold nanorods convert near-infrared radiation (NIR) into thermal energy, which can cleave a protector molecule linking the gRNA to the nanorod or induce Cas9 expression under a heat-sensitive promoter. Both mechanisms have been shown to successfully disrupt the *EGFP* gene, significantly reducing the fluorescence of edited A549 or HEK293T cells in comparison to cells treated with the nanorod and not exposed to near-infrared radiation (64,65). Upconversion nanoparticle (UCNP)-mediated conversion of NIR to UV radiation can similarly induce CRISPR/Cas9 release when the UCNP-encapsulated plasmid or RNP is conjugated with a UV-sensitive polyelectrolyte or UV-cleavable compound, respectively. These systems have both been shown to successfully disrupt the *Plk1* gene and inhibit tumor progression *in vivo* (66,67). Magnetic nanoparticles have also been engineered such that the application of an external magnetic field can specifically guide the nanoparticles to the target cells and induce CRISPR/Cas9 release. *In vitro* findings suggest that these nanoparticles may deliver CRISPR/Cas9 RNA across the blood-brain barrier for applications such as the eradication of latent HIV-1 reservoirs (68). Nanoliposomal particles conjugated with microbubbles can also be engineered to oscillate in response to ultrasound, disrupting the cell membrane and increasing permeability to the CRISPR/Cas9 material. This system has been used to locally deliver a Cas9/gRNA RNP to the dermal papilla cells in mice with androgenic alopecia. CRISPR/Cas9-mediated SRDA52 knockout effectively restored hair growth in these mice (69). Table 4 summarizes the major types of nanoparticle-based delivery systems explored for genome editing research.

Table 4: Common Features of Nonviral Systems Used in CRISPR/Cas9 Delivery[a]

Material	Function	Mechanism	Ref.
Cationic lipids (e.g. DOTAP, DOTMA, DOSPA, BHEM-Chol)	CRISPR/Cas9 encapsulation	Electrostatic interaction with anionic DNA, RNA, or RNP; often co-formulated with “helper” lipids such as DOPE and cholesterol	(42,70)
Cationic polymers (e.g. PEI, PAMAM, chitosan, PBAE)	CRISPR/Cas9 encapsulation; chitosan and PBAE provide biodegradability	Electrostatic interaction with anionic DNA, RNA, or RNP	(42,57, 58)
Lipids-polymer complexes	Modulation of nanoparticle surface and particle organization	Hydrophilic polymers provide a steric protection over the surface of the lipid nanoparticles	(52-54)
Zeolitic imidazolate frameworks	CRISPR/Cas9 encapsulation; biodegradability	Electrostatic interaction with anionic DNA, RNA, or RNP; decomposes in the low pH of the endosome	(55)
Bioreducible lipidoids	CRISPR/Cas9 encapsulation; biodegradability	Electrostatic interaction with anionic DNA, RNA, or RNP; disulfide bonds are cleaved in the cytosol	(59)
Gold	Loading of CRISPR/Cas9 material in nanoparticle core	Conjugated to thiolated DNA, then modified with additional materials (e.g. HDR donor, RNP, arginine, PAsp(DET), HIV-1 TAT peptide)	(60-63)
Gold nanorods	NIR controlled release	Conversion of NIR to thermal energy, activation of heat-sensitive promoter to express Cas9	(64,65)
Up-conversion nanoparticles	NIR controlled release	Conversion of NIR to UV radiation; modification of a UV-sensitive molecule conjugated to the plasmid or RNP induces release	(66,67)
Magnetic nanoparticles	Magnetic controlled release	External magnetic field guides the nanoparticle to the target cells and induces CRISPR/Cas9 release	(68)
Microbubble-conjugated nanoliposomal particles	Ultrasound controlled release	Ultrasound-induced oscillation of the microbubble disrupts the cell membrane, transiently increasing permeability and enabling uptake	(69)

[a] DOTAP: Dioleoyl-3-trimethylammonium propane; DOTMA: 1,2-di-O-octadecenyl-3-trimethylammonium propane; DOSPA: 2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propaniminium trifluoroacetate; BHEM-Chol: N,N-bis(2-hydroxyethyl)-N-methyl-N-(2-cholesteryloxycarbonyl aminoethyl)ammoniumbromide; DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; PEI: Polyethylenimine; PAMAM: Poly(amidoamine); PBAE: poly-beta amino esters; TAT: Trans-Activator of Transcription; NIR: Near-Infrared Radiation; HDR: Homology Directed Repair; PAsp(DET): poly{N'-[N-(2-aminoethyl)-2-aminoethyl]aspartamide}

5. THERAPEUTIC APPLICATIONS OF CRISPR/CAS9 GENOME EDITING

A. Monogenic Diseases

Nucleotide Repeat Expansion Disorders

CRISPR/Cas9 deletion strategies have been explored to cure nucleotide repeat expansion disorders such as Huntington's disease (HD). HD occurs due to a CAG repeat expansion in the huntingtin gene (*HTT*), which results in the toxic accumulation of an elongated huntingtin protein within neurons and gradual neurodegeneration (71). Because the mutation is autosomal dominant, a mutant allele (*mHTT*)-specific gene-editing strategy is preferable for preventing damage to the wild type allele. For example, paired gRNAs that target PAM sequences containing *mHTT*-specific single nucleotide polymorphisms (SNPs) will excise a large segment of *mHTT*. In two 2017 studies, this approach was taken and the results showed significant decrease in *mHTT* expression in iPSCs and mice (72,73). In 2019, Dabrowska et al. achieved similar deletions in HD patient-derived fibroblasts using Cas9 nickase capable of introducing a single-strand cut with the same specificity as a regular CRISPR/cas9 nuclease (74), reducing *mHTT* production by 70%. This treatment preserved the wild type *HTT* transcript produced from the edited allele, suggesting a potential advantage of the Cas9 nickase over the Cas9 nuclease, which, in contrast, may cause nonsense mutations that inhibit mutant huntingtin production but also prevent the synthesis of normal huntingtin (75).

A similar deletion approach has also been explored to treat myotonic dystrophy type 1 (DM1), an inherited disorder characterized by skeletal muscle pain and weakness, myotonia, and insulin resistance. In DM1, CTG repeat expansion mutations in the myotonic dystrophy protein kinase gene (*DMPK*) cause toxic accumulation of elongated mRNA transcripts into nuclear foci (76). These foci bind to muscleblind-like protein 1 (MBNL1), resulting in a loss of MBNL1 function and subsequent RNA splicing errors (77). Dastidar et al. (2018) and Lo

Scrudato et al. (2019) found that dual gRNA-mediated excision of the *DMPK* repeat expansion relocalized and reestablished RNA splicing patterns *in vitro* and *in vivo* (78,79). However, this strategy lacks allele specificity, and other studies have reported undesired mutations in both the mutant and wild type allele (80,81). Nuclease-dead Cas9 (dCas9) and one gRNA targeting the CUG expansion in the mutant *DMPK* RNA transcript could produce similar therapeutic results while preventing off-target mutagenesis. In this system, termed RCas9, targeted RNA cleavage is achieved by fusing dCas9 to the PIN RNA endonuclease domain of telomerase-binding protein EST1A (82). In 2020, Batra et al. found that AAV-RCas9-gRNA targeting the *DMPK* transcript relocalized MBNL1 and corrected RNA splicing abnormalities in DM1 mice (83).

Inherited Bleeding Disorders

CRISPR/Cas9-mediated gene insertion is applicable for treating Hemophilia A and B, characterized by mutation-induced deficiencies in clotting factors 8 (FVIII) and 9 (FIX), respectively (84).

Hemophilia A is treatable by inserting a functional FVIII cDNA at a safe genomic locus. In 2019, three studies published by Sung et al., Chen et al., and Park et al. demonstrated efficient FVIII insertion at intron 1 of the FVIII locus, intron 13 of the albumin (Alb) locus, and the intergenic H11 locus, respectively (85-87). Sung et al. and Chen et al. both used the B-domain deleted form of FVIII to reduce the DNA fragment size and facilitate packaging into an AAV vector. Sung et al. reported a gene insertion frequency of 81.8% in patient-derived iPSCs and a 2.9-fold increase in FVIII activity in the differentiated endothelial cells compared to the control (85). Chen et al. utilized a dual AAV8 vector system to deliver the SaCas9/gRNA plasmid and the FVIII gene in C57BL/6 mice and observed a dose-dependent increase in plasma FVIII activity. They also observed dose-dependent improvements in the activated partial thromboplastin time and the results of the tail vein bleeding assay. At a dose of 3×10^{12} vg/kg, these phenotypic results were very close to those observed in the wild-type mouse (86).

Hemophilia B is similarly treatable by inserting functional FIX cDNA into the FIX locus. In Hemophilia B mice, Ohmori et al. (2017) successfully employed a dual AAV8 vector system to insert FIX cDNA at intron 1 of the FIX locus, increasing FIX production to 11.7-39.6% at a high dose and reducing the aPTT by approximately 20 seconds (88). With the goal of achieving prolonged expression of functional FIX, Wang et al. (2019) inserted a FIX cDNA carrying the Padua mutation, which enhances FIX activity, into exon 2 of the FIX locus in neonatal and adult mice. FIX expression increased to about 10-15% of the normal level, and FIX activity increased to about 150-200% of the normal level. These elevated FIX expression and activity levels persisted for 8 months after a single injection and 24 weeks after partial hepatectomy (89).

Duchenne Muscular Dystrophy

Multiple CRISPR/Cas9 gene-editing strategies are applicable for treating Duchenne Muscular Dystrophy, a disorder caused by a frameshift mutation in the Duchene muscular dystrophy gene (*DMD*) that produces a truncated and dysfunctional form of dystrophin. The mutant dystrophin protein is degraded in the cell, causing increased muscle tissue stress and damage. Such mutations tend to occur in “hotspot” regions between exons 2-20 and exons 44-53 (90). These exons are targetable with antisense oligonucleotides, which bind to the mutant exon in the pre-mRNA, excluding the exon during RNA splicing (91). This effect, known as exon skipping, restores the reading frame, producing a functional truncated dystrophin protein and milder disease phenotype known as Becker Muscular Dystrophy (92). Antisense oligonucleotides create this effect transiently, whereas CRISPR/Cas9-mediated deletion of the mutant exon or disruption of the adjacent exon splice site enables permanent exon skipping.

Ideally, HDR-mediated replacement of the mutant *DMD* gene with a healthy copy could restore the full-length dystrophin and reverse the disease phenotype. However, HDR efficiency currently limits the practicality of this strategy. Therefore, studies have focused primarily on permanent exon skipping. Mutant exon deletion requires paired gRNAs, whereas the splice

acceptor site disruption only requires one gRNA and induces the desired effect more efficiently. In 2017, Amoasii et al. corrected an exon 50 deletion mutation using an sgRNA targeting a region adjacent to the splice acceptor site of exon 51, restoring up to 90% of dystrophin expression in both mouse and canine models (93,94). Ifuku et al. (2018) and Min et al. (2019) both successfully corrected an exon 44 deletion mutation by disrupting the splicing acceptor site in exon 45 in iPSC-derived myoblasts and patient iPSC-derived cardiomyocytes, respectively (95,96).

Inherited Retinal Dystrophies

CRISPR/Cas9 is also suitable for treating retinitis pigmentosa (RP). Mutations in RP cause the gradual loss of photoreceptor cells in the retina, adversely affecting night and peripheral vision and eventually causing blindness (97). Among many genes associated with RP, the *RHO* gene encoding rhodopsin is the most common (98). Many different point mutations in the *RHO* gene can cause misfolding and toxic accumulation of rhodopsin in the late endosome (99). Although CRISPR/Cas9 can disrupt specific *RHO* mutations, the number of *RHO* mutations implicated in RP renders this strategy impractical. Despite the limited efficiency of HDR, Tsai et al. (2018) successfully corrected three different *RHO* mutations in mice using a universal HDR deletion and replacement strategy, reporting a 17-36% increase in the thickness of the outer nuclear layer (ONL) compared to gene replacement-only therapy (100). A similar correction strategy has been applied for *PDE6B*, which causes an autosomal recessive form of RP (98). In 2019, Vagni et al. corrected mutant *PDE6B* by HDR. In the retina of mice carrying the mutation, the editing efficiency was about 0.164%, and was sufficient to preserve the a and b waves in the electroretinography test and significantly improve visual acuity (101). Cai et al. (2019) increased the HDR efficiency by 1.7-fold by incorporating bacterial recombinase A (RecA), an ATP dependent DNA binding protein that catalyzes reactions in homologous recombination, into the CRISPR/Cas9 system. Cas9/RecA-treated mice showed a five-fold

increase in the number of surviving rods and a four-fold increase in the number of surviving cones compared to Cas9-treated mice. Cas9/RecA-treated mice also showed significant improvements in the ERG signals and the pupillary light reflex (PLR) compared to Cas9 treated mice (102).

A promising clinical application for CRISPR/Cas9 has emerged for the treatment of Leber Congenital Amaurosis 10 (LCA10), which causes severe vision loss at birth (103). The most common mutation implicated in LCA10 is the IVS26 point mutation in intron 26 of the *CEP290* gene. This mutation generates a novel splice donor, creating a 128 bp pseudoexon in the coding sequence. As a result, the production of functional CEP290 protein significantly decreases, causing abnormalities in the retinal cilia (104). In contrast to the *RPE* gene, which can be packaged into an AAV vector and delivered to treat LCA2, the 8kb size of the *CEP290* coding sequence limits the development of similar LCA10 gene therapy (105). Therefore, CRISPR/Cas9 approaches for LCA10 treatment have become favorable. In 2019, Maeder et al. developed a therapeutic called EDIT-101, consisting of paired gRNAs designed to excise the IVS26 mutation. They successfully demonstrated that an AAV5 vector could localize SaCas9 to the photoreceptors and that EDIT-101 could correct the ISV26 mutation at a therapeutically relevant levels in mouse and non-human primate models (106), leading to FDA authorization of clinical testing (Table 5).

Inherited Red Blood Cell Disorders

CRISPR/Cas9 also has clinical applications in treating inherited red blood cell disorders caused by mutations in the hemoglobin subunit beta gene (*HBB*) encoding the β subunit of hemoglobin. In sickle cell disease, a single Glu6Val mutation in the *HBB* gene generates an abnormal hemoglobin molecule known as hemoglobin S, which deforms red blood cells into a sickle shape (107). These misshapen blood cells can break down rapidly or become stuck in a

blood vessel (108). In β-thalassemia, over 300 mutations in the *HBB* gene can reduce or prevent β-globin production (109).

While HDR-mediated replacement of the mutant *HBB* gene with a healthy copy can restore functional hemoglobin production *in vitro* (110), the *in vivo* efficacy of this approach remains unestablished. A novel CRISPR/Cas9 therapeutic known as CTX001, based on a different editing strategy, has reached phase 1/2 clinical testing for SCD and β-thalassemia. This strategy targets *BCL11A*, a gene encoding a transcription factor that typically suppresses fetal hemoglobin production in adults. Disrupting *BCL11A* in patient-derived hematopoietic stem cells (HSCs) *ex vivo* and engrafting the edited cells back into the patient may restore fetal hemoglobin synthesis, compensating for the deficiency in adult hemoglobin (111). As of 2020, 5 patients in the phase 1/2 trial of CTX001 have been dosed and successfully engrafted, and CRISPR Therapeutics and Vertex have published data for the first two β-thalassemia patients treated. Fifteen months after treatment, patient 1 had total hemoglobin levels of 14.2 g/dL, fetal hemoglobin of 13.5 g/dL, and 100.0% of erythrocytes expressing γ-globin. Five months after treatment, patient 2 had total hemoglobin levels of 12.5 g/dL, fetal hemoglobin of 12.2 g/dL, and 99.4% of erythrocytes expressing γ-globin. The FDA has granted CTX001 fast track designation and the designation of regenerative medicine advance therapy (112). Table 5 provides a brief summary on genome editing-based treatments for monogenic diseases.

Table 5. Summary of the Current Applications of CRISPR/Cas9 Gene Editing in the Treatment of Monogenic Diseases[a]

Disease Name	Genetic Cause	CRISPR/Cas9 Editing Strategy	Stage of Testing
Huntington's Disease	CAG repeat expansion in *HTT* gene	Allele-specific deletion of excess CAG repeats using Cas9 nuclease or nickase	*In vitro*
Myotonic Dystrophy Type 1	CTG repeat expansion in *DMPK* gene	Deletion of excess CTG repeats using Cas9 nuclease, Cas9 nickase, or dCas9	*In vitro* and *in vivo*
Hemophilia A	FVIII deficiency	Insertion of functional FVIII gene copy	*In vitro* and *in vivo*
Hemophilia B	FIX deficiency	Insertion of functional FIX gene copy	*In vitro* and *in vivo*
Duchenne Muscular Dystrophy	Frameshift mutation in *DMD* gene	Deletion of mutant exon Large deletion of mutation "hotspot" region Disruption of exon splice acceptor site	*In vitro* and *in vivo*
Retinitis Pigmentosa	Autosomal dominant mutation in *RHO* gene	Disruption of the *RHO* gene mutation	*In vitro* and *in vivo*
		Replacement of mutant *RHO* gene with functional copy	*In vivo*
	Autosomal recessive mutation in *PDE6B* gene	Replacement of mutant *PDE6B* gene with a functional copy	*In vivo*
Leber Congenital Amaurosis 10	IVS26 mutation in *CEP290* gene	Deletion of the pseudoexon	Clinical, EDIT-101, NCT03872479
Sickle Cell Disease	Glu6Val mutation in *HBB* gene	*Ex vivo* disruption of *BCL11A* in HSCs to induce HbF production	Clinical, CTX001, NCT03745287
β-thalassemia	Various mutations in *HBB* gene		

[a]BCL11A: B-cell lymphoma/leukemia 11A; DMPK: DM1 protein kinase; HBB: Hemoglobin subunit beta; HBF: fetal hemoglobin; HSC: hematopoietic stem cells; PDE6B: Phosphodiesterase 6B; RHO: Rhodopsin

[b] Assembled based on the information taking from https://www.clinicaltrials.gov/

B. Viral Infections

Herpes Simplex Virus 1

CRISPR/Cas9 gene editing may cure latent viral infections impossible to eliminate with current antiviral therapies, which only target actively replicating virions. For example, herpes simplex virus 1 (HSV-1) initially infects epithelial cells and is transported to sensory neurons, where the virus establishes a latent reservoir. The linear dsDNA genome remains present episomally, and the latency-associated transcript represses gene transcription (113).

In 2016, van Diemen et al. identified 12 essential HSV-1 genes and designed 4 gRNAs against each gene. A combinatorial gRNA approach using gRNA pairs targeting *UL29* and *UL8* or *UL29* and *UL52* blocked HSV-1 replication and prevented viral escape in Vero cells (114). In 2021, Yin et al. used a lentiviral vector to deliver a CRISPR/Cas9 plasmid targeting *UL29* and *UL8* into the cornea of HSV-1 infected mice. This system effectively reduced HSV-1 viral loads to nearly undetectable levels in the cornea and trigeminal ganglion and prevented symptoms of herpetic stromal keratitis (115).

Human Immunodeficiency Virus 1

Retroviruses such as human immunodeficiency virus 1 (HIV-1) establish proviral latent reservoirs. When active, HIV-1 infects and kills $CD4^+$ T cells, suppressing the host immune response to secondary infections (116). During the infection cycle, reverse transcriptase converts the RNA genome into cDNA which is then integrated into the host cell genome and transcriptionally silenced (117).

Early studies conducted in 2015 demonstrated that disruption of HIV-1 genes and long terminal repeats (LTRs) eliminates the virus and increases the viability of latently infected T cells (118,119). Subsequent studies have found that, multiplexed gRNAs targeting non-overlapping sequences inhibit HIV-1 infection and prevent viral escape more effectively than a single gRNA or multiplexed gRNAs with overlapping target sequences (120-122,123). In 2019, Dash et al.

demonstrated that sequential administration of long-acting antiretroviral drugs and a multiplexed AAV9-CRISPR/Cas9 system targeting the long-terminal repeats and the *gag* gene synergistically prevented the loss of CD4+ T cells in infected mice. Moreover, the dual treatment prevented viral rebound in 3 out of 6 treated mice, and reduced the levels of HIV-1 DNA and RNA below the detection limit in the analyzed tissues and plasma of two mice (124).

Human Papillomavirus

Approximately 70% of cervical cancers are associated with human papillomavirus (HPV) subtypes 16 and 18. The DNA genome of HPV16/18 integrates into the infected epithelial cell genome. Constitutive expression of the HPV E6 and E7 oncogenes destabilizes the host genome and drives carcinogenesis (125). Therefore, the E6 and E7 genes are considered therapeutic targets for preventing cervical cancer.

Studies conducted by Zhen et al. in 2017 and 2018 have demonstrated that CRISPR/Cas9-mediated disruption of E6 and E7 synergistically inhibits cervical cancer growth when paired with either cisplatin or programmed cell death protein 1 gene knockout (126,127). In both studies, Zhen et al. injected the naked Cas9/gRNA plasmid for *in vivo* delivery. More recent studies have focused on testing more clinically applicable delivery systems. Ling et al. (2020) intratumorally injected PEGylated liposomes carrying the Cas9/gRNA plasmid in a xenograft mouse model, resulting in dose-dependent tumor growth inhibition and upregulation of tumor suppressor gene p53 and its downstream target p21 (128). Gao et al. also observed promising *in vivo* results using PBAE nanoparticles and two different hyperbranched PAMAM-PBAE copolymer (hPPC)-based nanoparticles encapsulating a Cas9/gRNA plasmid targeting HPV16 E7. PBAE, hPPC1, and hPPC2 suppressed tumor growth by 85.0%, 90.3%, and 80.5%, respectively. Moreover, each system upregulated tumor suppressor gene RB1 and downregulated cervical cancer markers Ki67 and CD34 (58).

Hepatitis B Virus

Hepatitis B virus (HBV) primarily infects hepatocytes. Its DNA genome can integrate into the host genome or persist episomally in the form of covalently closed circular DNA (cccDNA) (129). The episomal DNA of HBV remains transcriptionally active, resulting in chronic infection (130). Early studies conducted in 2015 found that disrupting conserved regions of the HBV genome with CRISPR/Cas9 inhibits infection in multiple HBV genotypes (131-133). Sakuma et al. (2016) also demonstrated effective multiplexed disruption of the *S*, *X*, and *C* genes *in vitro* (134).

Recent studies have reported varying efficacy of AAV vectors for *in vivo* HBV genome disruption. For example, Li et al. (2018) intravenously injected AAV8-SaCas9/gRNA targeting the HBV core region to HBV transgenic mice. They observed, 38 days after the injection, a significant decrease in the serum levels of HBsAg, HBeAg, and HBV cccDNA and the inhibition of HBcAg expression in hepatocytes (135). However, Liu et al. (2018), using a similar AAV8-SaCas9/gRNA system, failed to observe a significant reduction in serum HBsAG and HBV DNA. They concluded that complete *in vivo* suppression of HBV infection would likely require a very high AAV titer (136). In 2020, Stone et al. tested an AAV2-Cas9/gRNA system in chronically infected mice concurrently receiving entecavir treatment. They reported detectable HBV genome editing in five of the eight treated mice, but the decrease in hepatocyte cccDNA levels was not statistically significant compared to the control mice (137). However, Kayesh et al. (2020) conducted a similar study and observed a significant decrease in hepatic HBV DNA and cccDNA levels (138).

In 2021, Zhen et al. found that simultaneous CRISPR/Cas9 disruption of the HBV genome and the host PD-1 gene synergistically reduced serum HBsAg levels by about 90% and almost completely blocked the expression of HBsAg in the livers of mice hydrodynamically injected with the CRISPR/Cas9 plasmid. Moreover, the combination therapy increased the population of mature dendritic cells in the lymph nodes, stimulated the expression of

proinflammatory cytokines and immune-associated genes, and enhanced the populations of CD4+ and CD8+ T cells (139). Further studies will be needed to determine whether this system exhibits similar efficacy when delivered via a clinically relevant vector such as AAV. Table 6 summarizes the major characteristics of genome editing-based treatment of viral infections.

Table 6. A Summary of the Current Preclinical Applications of CRISPR/Cas9 Gene Editing in the Elimination of Viral Infections.

Virus	Viral Genome	Gene Targets for CRISPR/Cas9 Disruption	Stage of Testing	Ref.
Herpes Simplex Virus 1	Linear double-stranded DNA genome	*UL8*, *UL29*, *UL52*	*In vitro* and *in vivo*	(113,114)
Human Immunodeficiency Virus 1	Positive sense single-stranded RNA, reverse transcribed into cDNA	Long terminal repeats (LTR), *tat*, *rev*, *pol*, *gag*	*In vitro* and *in vivo*	(116-124)
Human Papillomavirus Subtypes HPV16 and HPV18	Circular double-stranded DNA genome	E6 and E7	*In vitro* and *in vivo*	(61, 125-128)
Hepatitis B Virus	Covalently closed circular DNA	*S, C,* and *X*	*In vitro* and *in vivo*	(129-139)

C. Gene Edited Cell Therapies

The most clinically relevant therapeutic application of CRISPR/Cas9 genome editing is the production of gene-edited T cells with enhanced anti-tumor efficacy and reduced immunogenicity. In 2020, Sichuan University completed a phase I clinical trial of CRISPR-edited T cells for metastatic non-small cell lung cancer. In patient-derived T cells, the *PDCD1* gene encoding programmed cell death protein 1 (PD-1), which downregulates T cell immune responses, was knocked out *ex vivo* (140). This trial verified the general safety of CRISPR/Cas9 edited T cells in humans, but failed to establish adequate therapeutic efficacy.

CRISPR/Cas9 gene editing has improved the safety and efficacy of CAR T cells engineered to express an artificial tumor cell-targeting receptor. CAR T cells are commonly produced by collecting T cells from the patient, then using a retroviral vector to transduce the cells with a gene that encodes a chimeric antigen receptor (CAR), which integrates into the host cell genome (141). CRISPR/Cas9 can similarly deliver a CAR-encoding gene while preventing off-target transgene integration. Moreover, CRISPR/Cas9 can disrupt *PD-1* and genes such as *TCR* and *B2M* that contribute to patient immune responses (142). This multiplexed gene editing approach enables efficient and scalable manufacture of universal allogeneic CAR T cells with enhanced anti-tumor activity. In 2020, CRISPR Therapeutics reported promising results from 11 patients with refractory B cell malignancies in a phase 1 clinical trial of CTX110, an allogeneic CAR T cell targeting the CD19 antigen. Patients were infused with fludarabine and cyclophosphamide 3 days before the start and then infused with CTX110. At dose levels 1 through 3, no cases of graft-versus-host disease or severe treatment-related adverse events were observed. CTX110 was detectable in all patients above dose level 2, and 2 of the 4 patients receiving dose level 3 achieved complete responses (143). Table 7 summarizes the currently active clinical trials for CRISPR/Cas9-edited CAR T cell therapy.

Table 7. Status of Currently Active Clinical Trials for CRISPR/Cas9 Edited CAR T Cell Therapy[a]

Trial Identifier	Disease Name	CASPR/Cas 19-Mediated Intervention	Phase	Status
NCT03545815	Mesothelin positive multiple solid tumors	CRISPR-Cas9 mediated PD-1 and TCR gene-knocked out CAR-T cells	1	Recruiting
NCT04035434	Relapsed or refractory B-cell malignancies	CD19-targeted allogeneic CAR T cells (CTX110)	1, 2	Recruiting
NCT04244656	Multiple myeloma	BMCA-targeted allogeneic CAR T cells (CTX120)	1	Recruiting
NCT04438083	Renal cell carcinoma	CD70-targeted allogeneic CAR T cells (CTX130)	1	Recruiting
NCT04502446	T cell lymphoma	CD70-targeted allogeneic CAR T cells (CTX130)	1	Recruiting

[a] Assembled based on the information taken from https://www.clinicaltrials.gov/

CAR-T: chimeric artificial receptor modified T cells; PD-1: programmed cell death protein 1; TCR: T cell receptor; BMCA: B cell maturation antigen; CD: cluster of differentiation

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The discovery and development of protein-based ZFN and TALEN systems and RNA-based CRISPR/Cas systems have made genome editing a viable approach for treatment of genetic, viral, and metabolic diseases caused by monogenic or multigenic factors. The CRISPR/Cas9 system, being easy to work with, has driven recent efforts in generating transgenic organisms and testing the feasibility of genome editing for disease treatment. While CRISPR/Cas9 genome editing has shown promising results for many therapeutic applications in cell culture and animal models, the clinical application of many CRISPR/Cas9-based therapeutics remains limited by barriers to safe and efficient systemic *in vivo* delivery. The only *in vivo* CRISPR/Cas9 system that has reached the clinical stage is EDIT-101, in which the delivery is local to the retina. Other delivery systems including physical, viral and nonviral systems, although effective in preclinical studies, require improvements in safety, target specificity, and delivery efficiency. Moreover, the potential for the occurrence of off-target genome edits may raise ethical questions about testing these systems in humans. While somatic genome editing therapeutics have progressed closer to the clinical stage, germline genome editing applications are further limited by additional ethical concerns about inducing heritable changes to the human genome and obtaining informed consent for treatment (144).

The relatively low efficiency of HDR currently limits therapeutic CRISPR/Cas9 applications based on gene insertion or replacement. Therefore, preclinical and clinical therapeutic gene editing strategies widely rely on NHEJ-mediated gene disruption or deletion. In diseases such as DMD, NHEJ may produce a less ideal phenotypic outcome than an HDR gene correction strategy. HDR efficiency may be enhanced by co-delivering Cas9 with HDR agonists or fusing Cas9 with a DNA replication inhibitor, or packaging the Cas9/gRNA RNP and the HDR donor within one delivery vehicle (145).

Despite these technical challenges and areas of necessary improvement, the emergence of genome editing as a novel strategy for disease treatment has irreversibly impacted the ways that we optimize human health.

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