

Review

Advances in CRISPR/Cas-based Gene Therapy in Human Genetic Diseases

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Abstract

CRISPR/Cas genome editing is a simple, cost effective, and highly specific technique for introducing genetic variations. In mammalian cells, CRISPR/Cas can facilitate non-homologous end joining, homology-directed repair, and single-base exchanges. Cas9/Cas12a nuclease, dCas9 transcriptional regulators, base editors, PRIME editors and RNA editing tools are widely used in basic research. Currently, a variety of CRISPR/Cas-based therapeutics are being investigated in clinical trials. Among many new findings that have advanced the field, we highlight a few recent advances that are relevant to CRISPR/Cas-based gene therapies for monogenic human genetic diseases.

Key words: CRISPR/Cas, Gene editing, Gene therapy, Human disease, Genetic disease

Introduction

The past 20 years have witnessed great progress in genome editing techniques, including meganucleases, zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) nuclease system. These tools hold great potential for treating human disease, especially genetic diseases beyond the reach of traditional approaches [1]. The CRISPR/Cas system has rapidly become the most popular genome editing platform due to its simplicity and adaptability [2-5].

The CRISPR/Cas system was originally discovered as a prokaryotic adaptive immunity system used to recognize and cleave invading nucleic acids [6-8]. Based on this prokaryotic system, scientists have engineered a series of CRISPR/Cas tools for genome editing in mammalian cells, with the list of CRISPR/Cas systems in use continuing to expand. The most commonly used Cas nuclease

comes from *Streptococcus pyogenes* (SpCas9), and belongs to the type II CRISPR system. SpCas9 was the first to be reprogrammed for genome editing in mammalian cells. For specific nucleotide sequence recognition, engineered SpCas9 relies on the guidance of a single-guide RNA (sgRNA). Typically, sgRNA is composed of a scaffold sequence that is bound by the Cas protein, and a custom-designed ~20 nucleotide spacer that defines the genomic target to be modified. Following hybridization of the spacer to a target genomic sequence that is positioned next to a protospacer adjacent motif (PAM), the target DNA is cleaved, leading to a double-strand break (DSB) [7-9]. The Cas-mediated DSB is subsequently repaired by cellular DNA repair machinery via homology-directed repair (HDR) or the non-homologous end joining (NHEJ) pathway. NHEJ can be used to produce insertions and deletions (indels) that disrupt or inactivate the target gene, while HDR can be used for precise nucleotide sequence modifications, such as

point mutation correction [10-12] (**Figure 1a**).

To date, CRISPR/Cas-based techniques have been applied in various cell types and organisms. For therapeutic genome editing to treat monogenic diseases, CRISPR has the potential to be used directly in patients (*in vivo*) or in human cells (*in vitro*). In this review, we focus on CRISPR strategies used to treat human monogenic diseases, and discuss the

challenges associated with these approaches.

Recent advances in CRISPR/Cas technology

Shortly after SpCas9 was applied in mammalian cells, other Cas9 proteins have been studied and developed as genome editing tools. For example, smaller Cas9 proteins derived from *Staphylococcus aureus* called SaCas9 [13] and *Neisseria meningitidis* called Nme2Cas9 [14] exhibit gene editing efficiency comparable to that of SpCas9. These smaller Cas9s are more amenable for *in vivo* delivery than the large SpCas9 (~4.3 kb).

CRISPR/Cas9 technological advances have also enabled various applications of nuclease-deficient Cas9s, which can bind a specific region of the genome without creating DSBs. For example, catalytically inactive dead Cas9 (dCas9) can be fused with various transcription regulatory domains to create CRISPR activators (CRISPRa) or inhibitors (CRISPRi) that activate or silence the expression of a target gene [15] (**Figure 1b**). dCas9 can also be used as a visualization tool. Chen and colleagues have used dCas9 fused to enhanced green fluorescent protein (EGFP) to visualize repetitive DNA sequences using one sgRNA, or nonrepetitive loci using multiple sgRNAs [16-18]. In addition, David R. Liu's group has fused D10A Cas9 nickase with either cytidine or adenine deaminase to generate cytidine base editors (CBEs) and adenine base editors (ABEs), respectively. CBEs and ABEs generate transitions between A•T and C•G base pairs without causing high levels of double-stranded DNA cleavage in the target genomic region. Importantly, the Liu's group has extended base editing to utilize H840A Cas9 nickase fused with reverse transcriptase to create prime editors (PEs), which can achieve all possible base-to-base conversions (12 in total), as well as targeted insertions and deletions without DSBs or donor DNA templates [19] (**Figure 1c**).

In addition to DNA editing, Feng Zhang's lab has reported that an RNA-targeting CRISPR system based on Cas13 can target and cleave specific strands of RNA, and subsequently developed strategies called REPAIR (RNA Editing for Programmable A to I Replacement) and RESCUE (RNA Editing for Specific C to U Exchange) to edit RNA [20, 21]. Thus, RNA editing with CRISPR can efficiently modulate target genes at the transcript level in a transient and PAM independent manner.

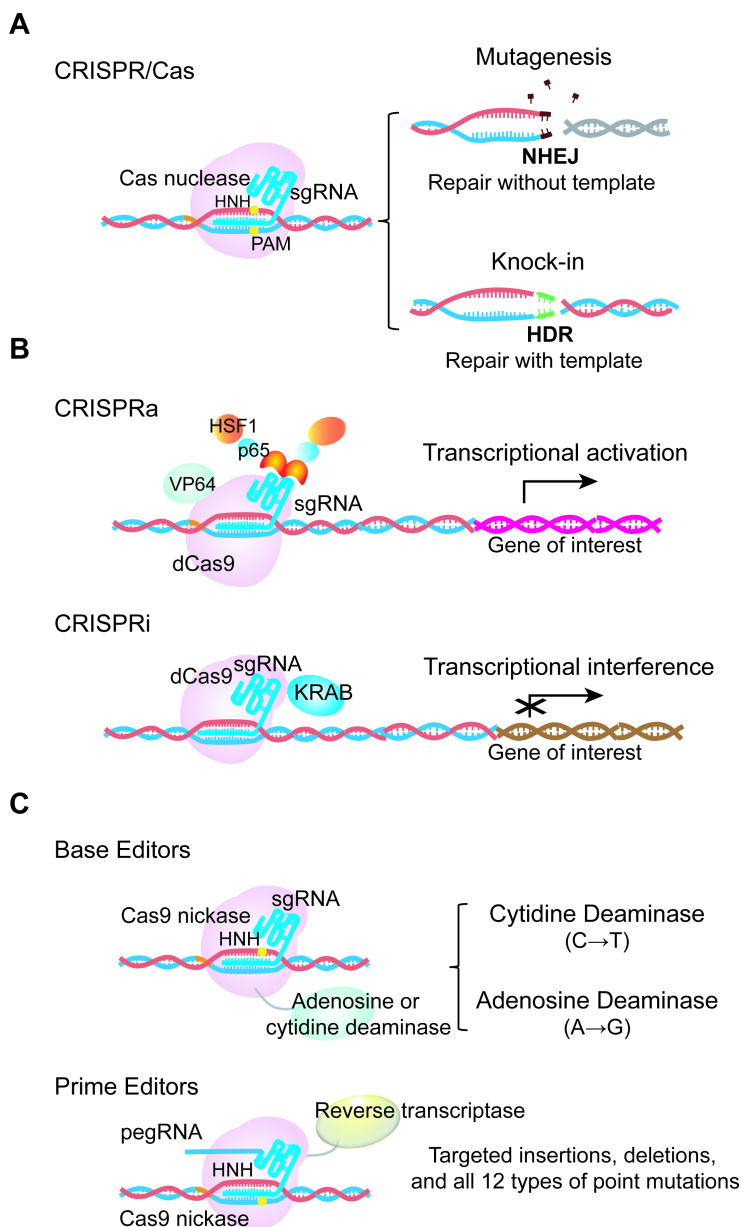


Figure 1. Examples of CRISPR/Cas9 technological advances. (a) Cas9 is directed by single guide RNA (sgRNA) to the target sequence. Double stranded DNA breaks are subsequently repaired by cellular DNA repair machinery via the NHEJ or HDR pathway. (b) dCas9 fused with transcriptional activators or repressors activates or inhibits the expression of a target gene. These systems are called CRISPRa or CRISPRi. dCas9 indicates catalytically inactive dead Cas9, which is able to bind the target DNA without cutting. CRISPRa, CRISPR activators to activate transcriptional process; CRISPRi, CRISPR inhibitors to interference transcriptional process. (c) Base editors are the combination of Cas9 D10A nickase with cytidine or adenine deaminase to induce G→T or A→G transition. Prime editor, different from base editors, is the fusion protein of Cas9 H840A nickase and reverse transcriptase. It can achieve up to 12 types of base-to-base conversions, and targeted insertions and deletions without DSBs or donor DNA templates. pegRNA, prime editing guide RNA.

This approach could provide a controllable approach for disease treatment.

Table 1. Animal diseases models generated by CRISPR listed in this review.

Corresponding human disease	Targeted gene	Substrate	Strategy	Author, year, (Refs)
Duchenne muscular dystrophy (DMD)	<i>DMD</i>	Human rhabdomyosarcoma cell line	NHEJ-mediated exon removal	Shimo et al, 2018,(31)
Aniridia-related keratopathy (ARK)	<i>PAX6</i>	Human limbal epithelial cells	NHEJ-mediated mutation	Roux et al, 2018, (32)
Osteogenesis Imperfecta (OI)	<i>COL1A1</i>	Human MCRii001-A iPSCs line	NHEJ-mediated a single base insertion	Far et al, 2019, (33)
X-linked adrenoleukodystrophy (X-ALD)	<i>ABCD1</i> & <i>ABCD2</i>	Murine BV-2 immortalized cell line	NHEJ-mediated gene deletion	Raas et al, 2019, (34)
Alzheimer's disease	<i>APPs</i> & <i>PSEN1M1</i>	Human and Mouse IPS Cell line	HDR-mediated mutation	Paquet et al, 2016, (35)
Duchenne muscular dystrophy (DMD)	<i>DMD</i>	Mouse	NHEJ-mediated exon removal	Egorova et al, 2019, (36)
Atherosclerosis	<i>LDLR</i>	Mouse liver	NHEJ-mediated gene deletion	Jarrett et al, 2018, (37)
Obesity (ob/ob) and diabetes (db/db)	<i>LEP</i> & <i>LEPR</i>	Mouse	NHEJ-mediated gene deletion	Roh et al, 2018, (38)
Resistance to thyroid hormone due to THRA mutation (RTHa)	<i>THRA</i>	Mouse	HDR-mediated mutation	Markossian et al, 2017, (39)
Alzheimer's disease (AD) and frontotemporal dementia (FTD)	<i>MAPT</i>	Mouse	NHEJ-mediated exon removal	Tan et al, 2018, (40)
Ryanodine receptor type I (RYR1)-related myopathies (RYR1 RM)	<i>RYR1</i>	Mouse muscle	HDR-mediated mutation	Brennan et al, 2019, (41)
Cystic fibrosis (CF)	<i>CFTR</i>	Sheep	NHEJ-mediated gene deletion	Fan et al, 2018, (42)
Diabetes mellitus (DM)	<i>PAX4</i>	Rabbit	NHEJ-mediated gene deletion	Xu et al, 2018, (43)
Huntington's disease (HD)	<i>HTT</i>	Pig	HDR-mediated exon fragments insertion	Yan et al, 2018, (44)
Autosomal recessive juvenile parkinsonism	<i>PINK1</i>	Monkey	NHEJ-mediated gene deletion	Yang et al, 2019, (45)

Applications of CRISPR in genetic diseases

To date, CRISPR/Cas systems have been used to investigate target genes in genome modification [22], splicing [23], transcription [24] and epigenetic regulation [25], and have been applied in a research setting to investigate and treat genetic diseases [26], infectious diseases [27], cancers [28], and immunological diseases [29, 30]. Among the exciting advances, translational use of CRISPR/Cas in monogenic human genetic diseases has the potential to provide long-term therapy after a single treatment. In this section, we summarize the recent applications of the CRISPR/Cas system in the generation of disease models and in the treatment of genetic diseases *in vitro* and *in vivo*.

Disease modeling using CRISPR/Cas

The generation of disease models is necessary for understanding disease mechanisms and developing new therapeutic strategies. CRISPR/Cas has been widely used for creating disease-related cellular models, such as DMD [31], aniridia-related keratopathy (ARK) [32], brittle bone [33], X-linked adrenoleukodystrophy (X-ALD) [34], and Alzheimer's disease [35]. Moreover, researchers have created a series of mouse models using CRISPR/Cas that recapitulate DMD [36], atherosclerosis [37], obesity and diabetes [38], RTHa [39], and Alzheimer's disease [40] (Table 1). One example is the development of a mouse model for ryanodine receptor type I-related myopathies (RYR1 RM), which harbors a patient-relevant point mutation (T4706M) engineered into one

allele, and a 16-base pair frameshift deletion engineered into the second allele of the RYR1 gene. Subsequent experiments demonstrated that this mouse model of RYR1 RM is a powerful tool for understanding the pathogenesis of recessive RYR1 RM, and for preclinical testing of therapeutic efficacy [41]. CRISPR/Cas has also been used to generate disease models in large animals, including sheep [42], rabbit [43], pig [44], and monkey [45]. For example, a monkey model was developed to study Parkinson's disease by introducing a *PINK1* deletion and revealed a requirement for functional *PINK1* in the developing primate brain [45]. CRISPR/Cas technology offers a flexible and user-friendly means of developing disease models to explore the genetic causes of diseases and evaluate therapeutic strategies.

Disease correction using CRISPR/Cas in model organisms and clinical trials

Monogenic diseases affect a large population of patients. In the ClinVar database, more than 75,000 pathogenic genetic variants have been identified [19, 46]. Here we summarize recent therapeutic applications of CRISPR/Cas in model organisms and in clinical trials (Table 2 and Table 3).

Hemoglobinopathies

Inherited blood disorders are good candidates for gene therapies because gene therapy can modify the causative gene in autologous hematopoietic stem cells (HSCs) and correct the hematopoietic system. β -thalassemia and sickle cell disease are two genetic blood diseases. β -thalassemia is due to various mutations including small insertions, single point

mutations or deletions in β -globin gene, resulting in loss or reduced β -globin synthesis [47]. Sickle cell disease is caused by a Glu→Val mutation in β -globin subunit of hemoglobin [48, 49], leading to abnormal hemoglobin S. Re-expressing the paralogous γ -globin genes is a universal strategy to ameliorate both β -globin disorders. The Bauer group applied CRISPR/Cas-based cleavage of the GATA1 binding site of the erythroid enhancer. This approach decreases erythroid expression of the γ -globin

repressor BCL11A and in turn increases γ -globin expression. This strategy is therapeutically practicable to produce durable fetal hemoglobin induction [50–52] (Table 2).

To date, three clinical trials aiming to treat patients with β -thalassemia and severe sickle cell disease by transfusion of CRISPR/Cas9 edited CD34+ human HSCs (CTX001) have been initiated by CRISPR Therapeutics in 2018 and Alllife Medical Science and Technology Co., Ltd in 2019 (Table 3).

Table 2. Preclinical CRISPR Therapy in disease models listed in this review.

Diseases	Target (Gene accession number)	Animal model or substrate	Delivery System	Strategy	Outcome	Author, year, (Refs)
β -thalassemia	<i>HBB</i> (NC_000011.10)	CD34+ HSPCs of β -thalassemia patients	RNP; electroporation	NHEJ-mediated mRNA splicing	93.0% indel frequency (SpCas9)	Xu et al, 2019 (50)
Hemoglobinopathies	<i>BCL11A</i> erythroid enhancer (NC_000002.12)	CD34+ HSPCs from sickle cell disease patient	RNP; electroporation	NHEJ-mediated enhancer disruption	54.6% reduction of <i>BCL11A</i> expression	Wu et al, 2019, (52)
Leber congenital amaurosis type 10	<i>CEP290</i> (NC_000012.12)	HuCEP290 IVS26 KI mouse eye	AAV; subretinal injection	NHEJ-mediated aberrant splicing	~60% editing rates in mice	Maeder et al, 2019, (53)
Duchenne muscular dystrophy (DMD)	<i>Dmd</i> (NC_000086.7)	mdx mice muscle	AAV; intramuscular injection (IM), retro-orbital injection (RO) and intraperitoneal injection (IP)	NHEJ-mediated mutant exon 23 skipping	~52% of WT (IP), ~71% of WT (RO), and ~70% of WT (IM) Dystrophin protein levels	Long et al, 2016, (55)
Duchenne muscular dystrophy (DMD)	<i>Dmd</i> (NC_000086.7)	mdx mice muscle	AAV; intramuscular injection	NHEJ-mediated mutant exon 23 skipping	~2% of all alleles from the whole muscle lysate	Nelson et al, 2016, (56)
Duchenne muscular dystrophy (DMD)	<i>Dmd</i> (NC_000086.7)	mdx mice muscle	AAV; intraperitoneal injection	NHEJ-mediated mutant exon 23 skipping	24–47% of total <i>Dmd</i> mRNA in cells including exon23 deletion	Tabebordbar et al, 2016, (57)
Congenital muscular dystrophy type 1A (MDC1A)	<i>Lama1</i> (NC_000083.6)	<i>dy²³/dy²³</i> mouse	AAV; intramuscular or tail vein injection	CRISPR activator mediated gene upregulation	3.6-fold upregulation of <i>Lama1</i>	Kemaladewi et al, 2019, (60)
Hereditary tyrosinemia type I (HTI)	<i>FAH</i> (NC_000073.6)	<i>FAH^{mut/mut}</i> mouse liver	AAV combined with lipid nanoparticles; intravenous injection	HDR-mediated point mutation correction	~0.8% initial correction rate in total liver DNA; more than 6% FAH+ hepatocytes	Yin et al, 2016, (62)
Hereditary tyrosinemia type I (HTI)	<i>FAH</i> (NC_000073.6)	<i>FAH^{mut/mut}</i> mouse hepatocytes	AAV; transplantation	HDR-mediated point mutation correction	2.6% alleles were corrected	VanLith et al, 2019, (63)
Hereditary tyrosinaemia type I (HTI)	<i>FAH</i> (NC_000073.6)	<i>FAH^{mut/mut}</i> mouse liver	plasmids; hydrodynamic tail-vein injection	Adenine base editor mediated point mutation correction	~0.3% initial correction rate in liver, ~4% FAH+ hepatocytes	Song et al, 2019, (64)
α 1-antitrypsin deficiency (AATD)	<i>AAT</i> (NC_000078.6)	<i>PiZ</i> mouse liver	AAV; intravenous injection	NHEJ-mediated mutant <i>AAT</i> disruption	~30% idel frequency	Bjursell et al, 2018, (66)
α 1-antitrypsin deficiency (AATD)	<i>AAT</i> (NC_000078.6)	<i>PiZ</i> mouse liver	AAV; intravenous injection	HdR-mediated point mutation correction	~2% correction rate in liver	Song et al, 2018, (67)
Perinatal Lethal Respiratory Failure	<i>SFTPC</i> (NC_000080.6)	<i>SFTPC^{173T}; R26^{tmTmc/+}</i> mouse fetus lung	adeno virus; intra-amniotic delivery	NHEJ-mediated mutant <i>SFTPC</i> disruption	~20% editing in the lung epithelium of fetuses	Alapati et al, 2019, (69)
Genetic Deafness	<i>Tmc1</i> (NC_000085.6)	Beethoven (Bth) mouse ear	AAV; Inner-ear injections	NHEJ-mediated mutant <i>Tmc</i> allele disruption	2.2% indel frequencies at 55 days after injection; 24% decrease in <i>Bth</i> mRNA	György et al, 2019, (75)

Table 3. CRISPR clinical trials for inherited diseases listed in this review.

Disease	Study title	Strategy	Study phase	Study type	Participants (No., Age)	Company	NCT Number	Website
Transfusion-Dependent β-thalassemia	A Safety and Efficacy Study Evaluating CTX001 in Subjects With Transfusion-Dependent β -Thalassemia	CTX001	Phase 1 Phase 2	Interventional	45 patients, ≥ 18 and ≤ 35 years of age	Vertex Pharmaceuticals Incorporated & CRISPR Therapeutics	NCT03655678	https://clinicaltrials.gov/ct2/show/NCT03655678
Sickle Cell Disease	A Safety and Efficacy Study Evaluating CTX001 in Subjects With Severe Sickle Cell Disease	CTX001	Phase 1 Phase 2	Interventional	45 patients, ≥ 18 and ≤ 35 years of age	Vertex Pharmaceuticals Incorporated & CRISPR Therapeutics	NCT03745287	https://clinicaltrials.gov/ct2/show/NCT03745287
β-thalassemia	iHSCs With the Gene Correction of HBB Intervent Subjects With β -thalassemia Mutations	HBB HSC-01	Early Phase 1	Interventional	12 patients, ≥ 2 and ≤ 60 years of age	Alllife Medical Science & Technology Co., Ltd.	NCT03728322	https://clinicaltrials.gov/ct2/show/NCT03728322
Leber congenital amaurosis LCA10	Single Ascending Dose Study in Participants With LCA10	AGN-1515 87	Phase 1 Phase 2	Interventional	18 patients, ≥ 3 Years	Allergan & Editas Medicine, Inc.	NCT03872479	https://clinicaltrials.gov/ct2/show/NCT03872479

Data from <https://clinicaltrials.gov/>

Inherited eye disease

Leber congenital amaurosis (LCA) is a rare genetic eye disease manifesting severe vision loss at birth or infancy. LCA10 caused by mutations in the *CEP290* gene is a severe retinal dystrophy. *CEP290* gene (~7.5 kb) is too large to be packaged into a single AAV. To overcome this limitation, Editas Medicine developed EDIT-101, a candidate genome editing therapeutic, to correct the *CEP290* splicing defect in human cells and in humanized *CEP290* mice by subretinal delivery. This approach uses SaCas9 to remove the aberrant splice donor generated by the IVS26 mutation. In the human *CEP290* IVS26 knock-in mouse model, over 94% of the treated eyes achieved therapeutic target editing level (10%) when the dose of AAV was not less than 1×10^{12} vg/ml [53]. Allergan and Editas Medicine have initiated a clinical trial of EDIT-101 for the treatment of LCA10 (Table 3).

Autosomal dominant cone-rod dystrophy (CORD6) is induced by a gain-of-function *GUCY2D* mutation. CRISPR/Cas components delivered by AAV specifically disrupt the early coding sequence of *GUCY2D* in the photoreceptors of mice and macaques by NHEJ. This study was the first to successfully perform somatic gene editing in primates using AAV-delivered CRISPR/Cas (up to 13% editing efficiency of *GUCY2D* mutant gene in macaque photoreceptor), and demonstrated the potential of CRISPR/Cas to cure inherited retinal diseases [54].

Muscular genetic disease

DMD, caused by mutations in the dystrophin gene, is the most common form of progressive muscular dystrophy, and is characterized by muscle weakness, loss of ambulation, and premature death. Several groups have used NHEJ to bypass a premature stop codon in exon 23 and restore the expression of dystrophin in neonatal and adult mice after local or systemic delivery of CRISPR/Cas components by AAV [55-57]. Similarly, CRISPR/Cas-induced NHEJ has been used to treat DMD in a DMD dog model after AAV-mediated systemic delivery of CRISPR gene editing components. 3 to 90% of dystrophin was recovered at 8 weeks after systemic delivery in skeletal muscle, the editing efficiency was dependent on muscle type and the muscle histology was improved in treated dogs [58]. In addition, ABE was delivered locally by intramuscular injection of a trans-splicing AAV to cure DMD in a mouse model [59]. These studies highlight the potential application of gene editing for the correction of DMD in patients.

Congenital muscular dystrophy type 1A (MDC1A), one of neuromuscular disorders, usually

appears at birth or infancy. It is mainly featured by hypotonia, myasthenia and amyotrophy. MDC1A is caused by loss-of-function mutations in *LAMA2*, which encodes for laminin- α 2. To compensate for the loss of laminin- α 2, Ronald D. Cohn and his colleagues used CRISPRa to upregulate *LAMA1*, which encodes laminin- α 1 and is a structurally similar protein to laminin- α 2. Upregulation of *LAMA1* ameliorates muscle wasting and paralysis in the MDC1A mouse model and provides a novel mutation-independent approach for disease correction [60].

Genetic liver disease

Hereditary tyrosinemia type I (HTI) patients with loss of function *FAH* mutations accumulate toxic metabolites that cause liver damage. CRISPR/Cas-mediated HDR has been used to correct *FAH*^{mut/mut} in the HTI mouse model by hydrodynamic injection of plasmids encoding CRISPR/Cas components or by combined delivery of AAV carrying HDR template and sgRNA and of nanoparticles with Cas9 mRNA [61, 62]. VanLith et al. transplanted edited hepatocytes with corrected *FAH* into recipient *FAH*-knockout mice and cured HTI mice [63]. Song et al. have used ABE in an adult mouse model of HTI to correct a *FAH* point mutation [64]. In addition to correcting *FAH*, several groups have knocked out hydroxyphenylpyruvate dioxygenase (HPD), which acts in the second step of tyrosine catabolism and is an upstream enzyme of *FAH*, to prevent toxic metabolite accumulation and treat HTI metabolic disease [65].

Patients with alpha-1 antitrypsin deficiency (AATD) develop liver disease due to a toxic gain-of-function mutant allele, as well as progressive lung disease due to the loss of AAT antiprotease function. CRISPR/Cas-mediated NHEJ has been used to disrupt mutant AAT to reduce the pathologic liver phenotype [66], while HDR has been used to correct an AAT point mutation [67].

Congenital genetic lung disease

Congenital genetic lung diseases include cystic fibrosis and inherited surfactant protein (SP) syndromes [68]. Monogenic lung diseases caused by mutations in SP genes of the pulmonary epithelium show perinatal lethal respiratory failure death or chronic diffuse lung disease with few therapeutic options. Using a CRISPR fluorescent reporter system, scientists precisely timed intra-amniotic delivery of CRISPR/Cas9 components into a prenatal mouse model with the human SP gene *SFTPC*^{I73T} mutation to inactivate mutant *SFTPC*^{I73T} gene through NHEJ. Prenatal gene editing in *SFTPC*^{I73T} mutant mice rescued lung pathophysiology, improved lung development, and increased survival rate to 22.8%. For intra-

amniotic delivery, the amniotic cavity of embryonic day 16 mouse fetus, in which fetal breathing movements are optimal for fetal lung editing, was injected. After prenatal CRISPR delivery, embryonic day 19 fetus achieved up to 32% SFTPC wild-type airway and alveolar epithelial cells in *SFTPC^{173T}* mice, rescued lung pathophysiology by immunohistology, improved lung development by reducing the synthesis of mis trafficked *SFTPC* mutant proprotein, and increased survival rate to 22.8% [69].

Cystic fibrosis is another life-threatening monogenic lung disease caused by mutations in *CFTR* gene [70]. Researchers applied CRISPR to precisely corrected *CFTR* carrying homozygous F508 deletion (F508del) in exon 10 in the induced pluripotent stem cells (iPSC) separated from cystic fibrosis patients [71] and the overall correction efficiency is up to 90% using *piggyBac* transposase as selection marker. Xu group applied the electroporation of CRISPR/Cas RNP and achieved more than 20% correction rate in patient-derived iPSC cell line with F508del mutation [72]. As expected, CRISPR-induced genetic correction leads to the recovery of *CFTR* function in airway epithelial cells or proximal lung organoids derived from iPSC.

Genetic deafness

At least half of all cases of profound congenital deafness are caused by genetic mutations and genetically inherited. Approximately 120 deafness-associated genes have been identified, but few treatments are available to slow or reverse genetic deafness [73]. Recently, David R. Liu's group employed cationic lipid-mediated *in vivo* delivery of Cas9-guide RNA complexes to disrupt the dominant deafness-associated allele in the humanized transmembrane channel-like 1 (*Tmc1*) Beethoven (*Bth*) mouse model and ameliorated the hearing loss in these animals [74]. David P. Corey's group screened 14 Cas9/sgRNA combinations and identified that SaCas9-KKH/gRNA could specially and safely recognize mutant *Tmc1* but not wildtype allele *in vitro* and *in vivo*, which provides a strategy to efficiently and selectively disrupt the dominant single nucleotide mutation rather than the wild-type alleles [75].

Overcoming limitations of CRISPR/Cas-based gene therapy

Extensive work is being done with CRISPR/Cas in disease research and recent reviews had summarized the advantages of CRISPR/Cas [76, 77]. The safety and efficacy of CRISPR/Cas9-based gene therapies need to be evaluated and refined before these therapies are applied in patients [78]. One of the common limitations for CRISPR/Cas is that not all the

mutation locus harbors the PAM motif, which the target recognition relies on. Besides, the challenges for using CRISPR/Cas as gene therapy include editing at off-target genomic sites, delivery vehicle, immunogenicity, and DNA damage response.

Off-target effects of CRISPR/Cas

Despite significant advances in understanding the CRISPR/Cas9 system, concerns remain regarding off-target effects. Indeed, several groups found a tradeoff between activity and specificity of CRISPR/Cas9, identifying off-target DNA cleavage by genome wide deep sequencing technique [79-81]. Moreover, CBEs and ABEs cause transcriptome-wide off-target RNA editing [82, 83]. Thus, unwanted off targets are concerns for the application of CRISPR. However, off-target effects can be reduced with sgRNA selection and optimization. Also, verification of *in vivo* off-targets (VIVO) can be used for defining and quantifying off-target editing of nucleases in whole organisms [84]. The recently developed anti-CRISPR proteins could conditionally control the activity of the CRISPR system [85-88], which may show the potential in reducing off-target effects. The development of more sensitive methods is necessary for detecting off-target editing at both genome and transcriptome levels.

In vivo delivery of CRISPR/Cas

AAV is the most widely used *in vivo* delivery of CRISPR/Cas. However, AAV has a limited packaging capacity, hindering all-in-one delivery of CRISPR/Cas components, in particular larger Cas-derived base editor and prime editor. This has led to continued development of smaller Cas9 orthologues like SaCas9 [13]. For instance, saCas9 or NmeCas9 and sgRNA have been combined into a single AAV vector for inducing indels to correct disease. For disease correction by HDR or base editors, dual AAV or split AAV vectors can be used to circumvent packaging size limitations [89, 90]. A disadvantage of such an approach is the requirement of uptake and expression of both AAV vectors into the same cell at roughly the same time to ensure intracellular Cas9:sgRNA complex formation.

CRISPR/Cas components can also be delivered by non-viral methods, for instance, Cas9 mRNA and sgRNA can be delivered to mouse liver by nanoparticles [62]. But the external and internal barriers for nanoparticles entering the cell and nucleus must be considered. Currently, nanoparticles carrying CRISPR/Cas components are largely applied to mice and delivered into liver. Because the liver contains fenestrated capillary endothelia. Further improvement of nanoparticle-based CRISPR/Cas

components delivery systems is needed for other target tissues.

Immune response stimulated by CRISPR/Cas

The application of CRISPR/Cas systems raises concerns over immunogenicity of the bacterially-derived Cas9 protein [91]. In a recent study, Charlesworth et al. demonstrated that anti-Cas9 responses are present in healthy human adults [92]. In 34 human blood samples, anti-Cas9 IgG antibodies were detected against SaCas9 (79% of samples), and against SpCas9 (65% of samples). The immunogenicity of SpCas9 in healthy humans has been reported by Michael's group. Specifically, they found that high prevalence of effector T cells towards SpCas9 exist prior to the delivery of SpCas9 [93]. This issue will need to be addressed in the clinical applications of CRISPR/Cas.

DNA damage response activated by CRISPR/Cas

In CRISPR/Cas gene editing via NHEJ and HDR, DSBs are generated at the target sites. DSB-based repair activates a p53-dependent DNA damage response and induces transient cell cycle arrest, leading to a decrease in efficiency of template-mediated precision genome editing [94]. In human pluripotent stem cells, p53-deficient cells are more susceptible to CRISPR-mediated modification [95]. These findings suggest that, during clinical trials, CRISPR-engineered cells or organs in patients should be monitored for p53 function. To avoid DSB triggered p53-mediated response, base editors (ABE and CBE) and prime editors can be applied for precision gene editing-mediated target gene correction.

Conclusion and perspectives of using CRISPR/Cas in the clinic

CRISPR/Cas has already shown great potential in generating disease models and correcting monogenic disease mutations. The CRISPR disease models can accelerate the discovery and development of drug targets. In addition to the widely used type II CRISPR/Cas systems, continued discovery and development of CRISPR systems from prokaryotic species has generated new technologies. For example, DN15-SpCas9 fusion protein blocks local NHEJ events and increases HDR frequency [96]. Moreover, Cas13a-based RNA-targeting tools enable RNA changes that are temporally and spatially controllable, and will broaden and facilitate the application of RNA therapy in human diseases. Before the application of CRISPR for human disease correction, efforts are needed to optimize and maximize the editing

efficiency as well as minimize off-targets and develop novel tools to specifically deliver the CRISPR components to the target tissue for gene editing [97, 98]. As CRISPR/Cas-based gene therapy enters clinical trials (Table 3), this technology holds great potential for treating genetic diseases particularly for the present incurable ones and enhancing cell therapies.

Abbreviations

ZFN, zinc finger nucleases; TALENS, transcription activator-like effector nucleases; CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated nuclease; SpCas9, Cas nuclease comes from *Streptococcus pyogenes*; sg-RNA, single-guide RNA; PAM, protospacer adjacent motif; DSB, double-strand break; NHEJ, non-homologous end joining; HDR, homology-directed repair; indels, insertions and deletions; CRISPRa, CRISPR activators; CRISPRi, CRISPR inhibitors; AAV, adeno-associated virus; CBEs, cytidine base editors; ABEs, adenine base editors; PE, prime editors; DMD, duchenne muscular dystrophy; HTI, hereditary tyrosinemia type I; AAV, adeno-associated virus.

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Competing Interests

The authors have declared that no competing interest exists.

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