REVIEW ARTICLE

Clinical applications of the CRISPR/Cas9 genome-editing system: Delivery options and challenges in precision medicine

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Abstract CRISPR/Cas9 is an effective gene editing tool with broad applications for the prevention or treatment of numerous diseases. It depends on CRISPR (clustered regularly interspaced short palindromic repeats) as a bacterial immune system and plays as a gene editing tool. Due to the higher specificity and efficiency of CRISPR/Cas9 compared to other editing approaches, it has been broadly investigated to treat numerous hereditary and acquired illnesses, including cancers, hemolytic diseases, immunodeficiency disorders, cardiovascular diseases, visual maladies, neurodegenerative conditions, and a few X-linked disorders. CRISPR/Cas9 system has been used to treat cancers through a variety of approaches, with stable gene editing techniques. Here, the applications and clinical trials of CRISPR/Cas9 in various illnesses are described. Due to its high precision and efficiency, CRISPR/Cas9 strategies may treat gene-related illnesses by deleting, inserting, modifying, or blocking the expression of

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specific genes. The most challenging barrier to the *in vivo* use of CRISPR/Cas9 like off-target effects will be discussed. The use of transfection vehicles for CRISPR/Cas9, including viral vectors (such as an Adeno-associated virus (*AAV*)), and the development of non-viral vectors is also considered.

Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR) was discovered in *Escherichia coli* and described as a 1664 nucleotide sequence by Ishino and colleagues in 1987.[1] Before its functional application, two critical studies on genes adjacent to the CRISPR locus[2] were done. The discovery of foreign viral DNA in CRISPR spacers[3] directly proposed the role of CRISPR as an innate immune system in bacteria to fight viruses.[4] This hypothesis was confirmed by experiments in Barango's laboratory, and the function of the CRISPR sequences was finally determined.[5] Due to its excellent ability to target and cut specific DNA sequences, CRISPR was widely introduced as a gene editing tool.[6] Compared to previous gene editing tools like zinc finger nucleases (ZFNs) and transcription-activating effector nucleases (TALENs), CRISPR-based gene-editing tools perform extremely better and are easier to handle.[7] Due to the development of this technique, Emmanuel Charpentier and Jennifer Doudna received the Nobel Prize in 2020.[8,9]

CRISPR-Cas systems are classified into two main classes and six sub-types. CRISPR-Cas9 as a member of type II is the simplest and most widely used in genetic research due to the presence of a single protein with endonuclease activity (Cas9). In this system, the Cas9 protein can target the desired sequence using an engineered guide RNA (gRNA) which makes a smooth double-stranded cut in three nucleotides sequence upstream at the target site which is called Protospacer-Adjacent Motif (PAM). PAM is located immediately downstream of the crRNA binding site of gRNA in the incomplete strand and is important in target recognition by Cas9. The PAM sequence (5′-NGG) is not present in CRISPR arrays and is only located on the foreign invading genome; this is the reason why Cas9 cannot cut the bacterial genome.[10,11]

As shown in Figure 1, intracellularly double-stranded breaks (DSBs) can be repaired either by non-homologous end joining (NHEJ),[12] a pathway that shows irregular break ends, or through Homology Directed Repair (HDR),[13] a pathway that requires homologous patterns, which is carried out in gene repair, addition, or replacement.[14] The wide potential of CRISPR lies in the directed action of Cas9 through an engineered sgRNA or crRNA/tracer RNA, allowing the researcher to target a wide range of genomic sequences.[15]

This technique has the potential to treat a large number of genetic diseases like cancers.[16,17] In this article, the current status of CRISPR applications in clinical trials and delivery methods will be reviewed. Finally, several challenges and opportunities facing researchers in this field will be elaborated.

Efforts to improve CRISPR/Cas9 technology

After the rapid development and remarkable success of this technique, researchers have tried tore-engineer and expand CRISPR/Cas9. CRISPR interference (CRISPRi), protein knockdown Cas9,[18] Cas9 nickase (nCas9), and dead Cas9 (dCas9) are some of these examples. Cas9 nickase (nCas9) has mutations in its nuclease domain which helps to create the cut in a single-stranded DNA.[19] A year after the creation of dead Cas9 (dCas9), the endonuclease-free Cas9 was designed to increase the quality of expression, particularly for CRISPRa.[20] Subsequently, more precise and reliable CRISPR/Cas9-based gene editing strategies without introducing DSBs, such as base editing[21,22] and primary editing[23] were developed. The base modification techniques, Cytidine Counting Base Editor (CBE) and Adenine Base Editor (ABE) typically consist of nCas9/dCas9 evolved cytidine deaminase/adenine deaminase. CBE promotes the conversion of CG to TA while ABE promotes the conversion of AT to GC, and the current established nNme2-CBE framework seems to have higher efficiency and flexibility.[24]

Ultimately, gene editing tools are still limited in creating specific base conversions. Therefore, some editing tools have been developed using changes in the transcriptions of dCas9. In this regard, gRNA primers were used to realize precise modification with less off-target effect.[23] With changes in CRISPR/Cas9 gene editing tools that are efficient and safe, they have the potential to enter clinical trials. A CRISPR-related transposase that not only cuts the DNA but also performs high-quality editing has been developed.[25] In terms of *in vivo* gene therapy, various CRISPR frameworks such as light- and chemical-activated Cas9 have been explored.[24,25] Also, CRISPR modifiers with smaller sizes have been investigated recently.[26]

Ex vivo genome editing

Ex vivo genome editing is a useful approach in which the genome of specific cells is altered *in vitro*, and then the modified cells are transplanted into the patient to induce a regenerative effect. This approach differs from *in vivo* genome modification approaches, where CRISPR/Cas9 or other genome-editing components are specifically delivered into the cell environment and perform their beneficial effect *in situ*.[27,28] Compared to the *in vivo* techniques, the *ex vivo* modification method requires more steps (such as
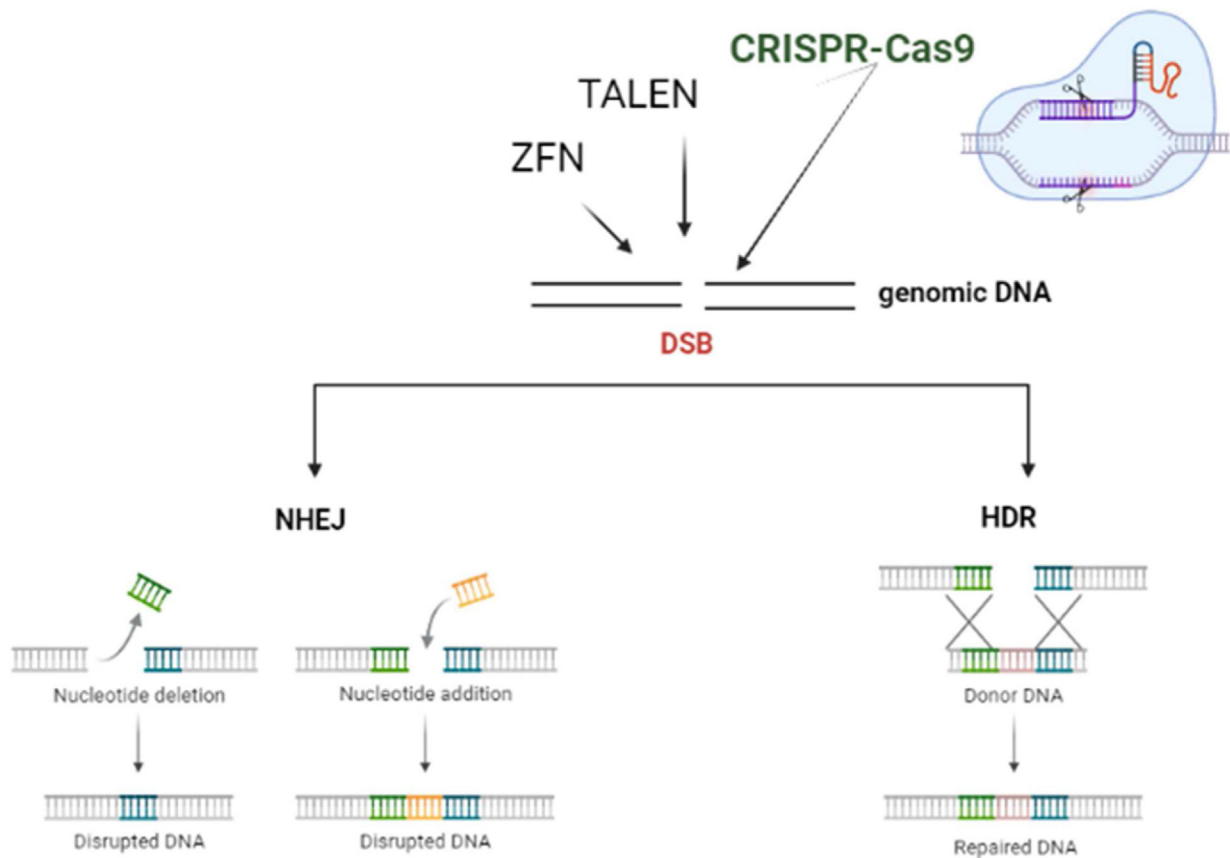
Figure 1 ZFNs, TALENs, and CRISPRs lead to DSBs in the genome and are repaired mainly by two pathways: (i) non-homologous end joining (NHEJ): error-prone; (ii) homology-based repair (HDR).

cell isolation, expansion, transfection, fixation, and transplantation) and may be more suitable for focusing on a specific organ rather than the whole organism.[29] Notably, it avoids the enormous challenges of *in vivo* transfection that have been widely discussed.[30,31] In addition, the *ex vivo* approach may have certain safety advantages, especially its low off-target rate. *In vivo* approaches should look after the unintended off-targets, either in the context of unintended transfer to non-target cell lines or in the form of unintended mutations in the genome. The *ex vivo* approach circumvents this issue by precisely altering the target cell, providing an opportunity to screen the effective alteration. In this context, the *ex vivo* applications of CRISPR/Cas9 for better genome editing and its methods and related references are summarized in Table 1.

Clinical trials involving *ex vivo* CRISPR-based genome editing

The primary clinical trial on CRISPR-based *ex vivo* genome editing endeavored to treat human immunodeficiency virus 1 (HIV-1) infection.[32] Disruption of *CCR5*, which encodes a critical receptor for the viral compartment, was initiated by nucleofection of *CCR5*-centered ribonucleoprotein complexes in patient-derived hematopoietic stem cells and progenitor cells (HSPCs). A 27-year-old man with HIV-1 disease and severe lymphoblastic leukemia was treated as

such which brought about effective and long-term engraftment of CRISPR-edited HSPCs. The efficacy of *CCR5* disruption increased from 5.2% to 8.3% in bone marrow cells over 19 months which did not reach the desired goal (ClinicalTrials.gov, NCT03164135). Recently, a clinical trial attempting to treat serious single-gene diseases with CRISPR-based genome editing systems with promising results has been reported.[33] Sickle cell disease and beta-thalassemia refer to a group of acquired hemoglobinopathies caused by changes in the gene of the beta-hemoglobin subunit (*HBB*) that resulted in misfolded beta-globin proteins. In this treatment approach, rather than the pathogenic *HBB* gene, BCL11A gene enhancer which is a translational variant to inhibit gamma-globin has been considered to re-establish fetal hemoglobin production, and compensate for the altered *HBB* gene. Patient-derived HSPCs were modified with CRISPR-Cas9 with a sgRNA targeting the *BCL11A* enhancer to create gene-edited HSPCs called CTX001. One subject with transfusion-dependent beta-thalassemia and one subject with sickle cell disease were examined with CTX001 after myeloablation. High altered allele frequencies and fetal hemoglobin levels were maintained, and both patients were cured of disease-related transfusion. However, actual adverse effects were observed in both patients like neutropenic pneumonia, hepatic vein-obstructive infection with sinus obstruction, sepsis with neutropenia, kidney stones, and gastric agony. This ongoing test is almost consistent with preliminary

Table 1 Clinical trials involving *ex vivo* or *in vivo* CRISPR-based genome editing.

Status	NCT Number	Diseases	Target gene	Intervention/treatment	Clinical phase	Delivery method
Completed	NCT04191148	Urinary tract infections	*E. coli* genome	LBP-EC01	Phase I	crPhage cocktail - *in vivo*
Unknown	NCT03728322	Thalassemia genetic diseases	Hemoglobin subunit beta (*HBB*)	No	Phase I	*ex vivo*
Active, not recruiting	NCT03655678	Thalassemia genetic diseases	BAF chromatin remodeling complex subunit 11 A (*BCL11A*)	CTX001	Phase II/III	Electroporation - *ex vivo*
Active not recruiting	NCT04205435	Beta-thalassemia	Hemoglobin subunit beta (*HBB*)	β-globin restored autologous HSC	Phase I/II	Electroporation - *ex vivo*
Enrolling by invitation	NCT05143307	HIV	Undisclosed	EBT-101	Phase I	AAV9 - *in vivo*
Concerned	2018-001320-19	Sickle cell disease, hematological diseases, hemoglobinopathies	*HBB*	CTX001	Phase I/II	Electroporation - *ex vivo*
Active	NCT03745287	Sickle cell disease, hematological diseases, hemoglobinopathies	BAF chromatin remodeling complex subunit 11 A (*BCL11A*)	CTX001	Phase II/III	Electroporation - *ex vivo*
Active	ChiCTR2100052858	Transfusion dependent beta-thalassemia	Undisclosed	RM-001	Phase I	Undisclosed - *ex vivo*
Active	NCT04037566	Leukemia lymphocytic acute in relapse, acute lymphocytic leukemia (ALL) refractory lymphoma, B-cell, CD19 positive, ALL	CD19 molecule, HPK1 hematopoietic progenitor kinase 1 (also known as *MAP4K1*)	Cyclophosphamide/ Fludarabine	Phase I	Lentivirus (LV) and electroporation - *ex vivo*
Active	NCT04560790	Herpes simplex virus refractory keratitis	*UL8*/*UL29*	BD111 adult single group dose	Phase I/II	mRNA transfection - *in vivo*
Active	NCT03872479	Blindness, Leber congenital amaurosis	Centrosomal protein 290 (*CEP290*)	EDIT-101	Phase I/II	Adeno-associated virus (AAV5) - *in vivo*
Active	NCT05210530	Type 1 diabetes	—	VCTX210A unit	Phase I	*Ex vivo*
Active	NCT05120830	Hereditary angioedema	Kallikrein B1 (*KLKB1*)	Biological NTLA-2002	Phase I/II	Lipid nanoparticles - *in vivo*
Active	NCT04601051	Hereditary transthyretin amyloidosis	Transthyretin (*TTR*)	NTLA-2001	Phase I	Lipid nanoparticles - *in vivo*

findings from additional trials (ClinicalTrials.gov, NCT03655678, and NCT03745287).

Furthermore, CRISPR-based *ex vivo* genome editing is additionally relevant to the treatment of malignant cancers.[34-36] The basic concept is to promote common antitumor responses of cytotoxic T cells by depleting the modulatory genes of some checkpoints via CRISPR-Cas9. Two clinical trials using this technique were recently published with promising results. In one of them, scientists attempted to treat various advanced and incurable cancers including multiple myeloma and liposarcoma by CRISPR-Cas9 genome editing.[34]

In their experiment, T cells were isolated from cancerous patients and engineered with CRISPR-Cas9 to suppress the endogenous T cell receptor (TCR) and immune-modulated cell transit protein 1 (*PD-1*). Specifically, deletion of the TCR α chain (*TRAC*), TCR β chain (*TRBC*), and *PDCD1* genes were initiated by electroporation of ribonucleoprotein complexes in patient-derived T cells. The modified T cells, called "*NYCE*" (CRISPR 3*X* NY-ESO-1-transduced cells) were then injected into patients intravenously. A total of 3 patients were treated with stable engraftment of edited T cells. The frequencies of target gene mutations in peripheral blood mononuclear cells were around 5%—10%, without significant off-target changes or actual adverse effects. Tumor recurrence was limited to the early stages of treatment, and all tumors inevitably progressed at the end of the trial (ClinicalTrials.gov, NCT03399448). In another comparative trial, non-small cell lung cancer was tried to be cured with CRISPR-engineered patient-derived T cells targeting the *PD-1* gene.[35]

PD-1 gene-associated exon disruption was induced by electroporation of Cas9-and sgRNA-encoding plasmids into patient-derived T cells. A total of 12 treated patients demonstrated the development of modified T cells, even though the average gene modification rate was very low (5.8%). No significant changes were seen outside the target or in real contrast situations. Clinically, two patients had stable disease at 8 weeks, all patients had an infection, and 11 patients had a progression of infection (ClinicalTrials.gov, NCT02793856).

In vivo genome editing

Initially reported with CRISPR-based *in vivo* gene editing, this innovation was linked to transthyretin amyloidosis, or *ATTR* amyloidosis, which resulted from the accumulation of misfolded transthyretin (*TTR*) protein in different organs and tissues.[37]

ATTR amyloidosis is a monogenic infection and almost all *TTR* proteins form amyloid fibrils in different organs and are an ideal target for CRISPR-based *in vivo* gene editing. NTLA-2001, a liver-trophic lipid nanoparticle framework containing Cas9 mRNA and sgRNA targeting human *TTR* gene was offered to decrease circulating TTR amyloid forms in humans. Six patients with TTR mutations and tactile polyneuropathy were treated with a single injection of NTLA-2001, which reduced serum *TTR* levels up to 52% in the low-injected dose group and 87% in the high-injected dose group after 4 weeks without significant side effects. To confirm the efficacy and long-term safety of the treatment, all patients were followed up (ClinicalTrials.gov, NCT04601051).

Although detailed information is not available, numerous research groups have tried to utilize CRISPR-based *in vivo* gene editing to treat Leber's innate amaurosis 10 as a monogenic infection disease (ClinicalTrials.gov, NCT03872479).

Clinical trials utilizing *ex vivo* and *in vivo* CRISPR-based gene editing is summarized in Table 1. Moreover, a broad investigation of animal models for advanced gene editing applications is reviewed in the following section.

Animal models in CRISPR applications

A lot of knowledge regarding CRISPR-based genome editing approaches is acquired through the implication of animal models. Some examples are referred to here. Leber's congenital amaurosis (*LCA*, OMIM #204000) comprises a group of early-onset retinal dystrophies of childhood, each subtype results from changes of distinctly different genes. *LCA* sort 10 (*LCA10*, OMIM #611755) is caused by a mutation in the gene of CEP290. CRISPR-based genome editing has been used in mouse *LCA10* models. Wild-type *CEP290* expression was successfully re-established by subretinal infusion of a single AAV encoding both SaCas9 and sgRNA.[38]

LCA2 (LCA2, OMIM #204100) is done by *RPE65* transfection. The rd12 mouse genetically edited with *LCA2* was under subretinal infusion with two AAVs encoding SpCas9, sgRNA, and donor DNA. The as-described mouse was effectively treated with subretinal infusion of adenine base editors utilizing RNPs,[39] intein-mediated part AAV vectors,[40] and prime editors utilizing trans-splicing part AAV vectors.[41] Retinitis pigmentosa (OMIM #268000), which alludes to a heterogeneous gathering of acquired visual infections that result in dynamic retinal degeneration, comprises 92 diverse phenotypes and is caused by changes in over 200 genes. AAV-mediated gene transfection to treat retinitis pigmentosa is one of the approved treatments in gene therapy history.[42]

Fundamental studies on CRISPR-based genome editing began in 2016. Since then, numerous CRISPR-based approaches (each focusing on different genes like Nrl Mertk, Pde6b, Rho, and RPGR),[43-49] have been done in animal models. In particular, the *Nrl* gene was terminated via NHEJ and inhibited by an approach called CRISPR blockades, both were mediated by AAV vectors. The Mertk gene was edited by a novel strategy called homology-independent targeted integration that focuses on integration. The Pde6b gene was modified via homology repair, and the Rho gene via NHEJ. These two later genes were activated by *in vivo* electroporation using Cas9-encoding plasmids. Hereditary tyrosinemia type 1 (*HT1*, OMIM #276700), a fatal inherited disorder caused by mutations in fumaryl acetoacetate hydrolase (FAH), results in the accumulation of deleterious metabolites leading to severe liver damage. CRISPR-based genome editing using human HT1 models in mice was initiated in 2014 which modulates pathogenic mutations.[50] Gene-edited hepatocytes showed a growth advantage over other mutated hepatocytes which can repopulate the liver in a truly regenerative manner. After this work, Cas9 modifications (NmeCas9,[51] St1Cas9[52]), base

editors,[53,54] and prime editors[41] have effectively protected the deadly HT1 phenotype in grown-up mouse models.

Phenylketonuria (PKU, OMIM #261600) is an autosomal recessive liver illness caused by mutations within the phenylalanine hydroxylase gene, which may cause mental impediment due to the neurotoxicity of metabolites. In grown-up mouse models, intravenous infusion of AAVs encoding an intein-split cytosine base editor effectively reestablished blood phenylalanine levels and switched the PKU-associated hide color.[55]

Afterward, the ordinary homology-directed repair approach reduced the side effects with the assistance of chemical modifiers.[56,57] Ornithine transcarbamylase (OTC) deficiency (OMIM #311250) is an X-linked metabolic clutter characterized by hyperammonemia and is caused by mutations within the OTC gene (OMIM *300,461). Employing a double AAV framework containing SaCas9-encoding sequences, sgRNA-encoding sequences, donor DNA, and OTC editions with homology-directed repair, lead to more survival in mouse models.[58]

Duchenne muscular dystrophy (DMD, OMIM #310200) is an acquired X-linked infection caused by changes within the dystrophin locus. The application of a CRISPR-based genome editing tool to regulate mutations and restore dystrophin expression in mouse zygotes was initiated in 2014.[59] Following this initial work, many other studies investigated the exact properties of CRISPR-Cas9 restoration of dystrophin expression in rat,[60–62] dog,[63] and pig[64] models of DMD. Furthermore, adenine base modification successfully reversed DMD pathology in mouse embryos and adult mouse models.[65]

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder in which the dynamic transmission of motor neurons is accompanied by loss of movement. Several causative features have been distinguished as early congenital ALS, and mutations in SOD1 (OMIM * 147,450) are believed to be initiators for most cases of ALS type 1 (ALS1, OMIM #105400). Recently, intravenous infusion of AAV encoding SaCas9 and SOD1-targeting sgRNA appeared to delay disease appearance and enhance the applicability in ALS mouse models.[66]

Glycogen capacity illness Ia (GSD1A, OMIM #232200), too known as von Gierke illness, is caused by pathogenic mutations within the glucose-6-phosphatase alpha subunit (G6PC) gene. This mutation causes glycogen accumulation all over the body. Recently, the profoundly dominant G6PC p. R83C mutation was subjected to CRISPR-based genome editing in vivo in mouse models using two AAVs; one encoding SaCas9 and the other encoding sgRNA[67] which knocked down the G6Pase gene.

Hutchinson–Gilford progeria disorder (HGPS, OMIM #176670) is caused by changes in the gene of layer A (LMNA). Recently, the LMNA c.1824C > T mutation was found in more than 90% of patients with HGPS. This was edited in transgenic mouse models using AAVs encoding conformations of the adenine base editor and resulted in more vascularization and life expectancy.[68] This report demonstrated the potential of modern genome editors to specifically create point mutations for the treatment of inherited disorders.

CRISPR/Cas9 delivery platforms

To fully exploit the editing potential of CRISPR/Cas9, they must be successfully delivered into target cells or tissues using appropriate vectors.[69] Various transfer methods are shown in Figure 2. In the following section, the advantages and disadvantages of each transfer method will be reviewed.
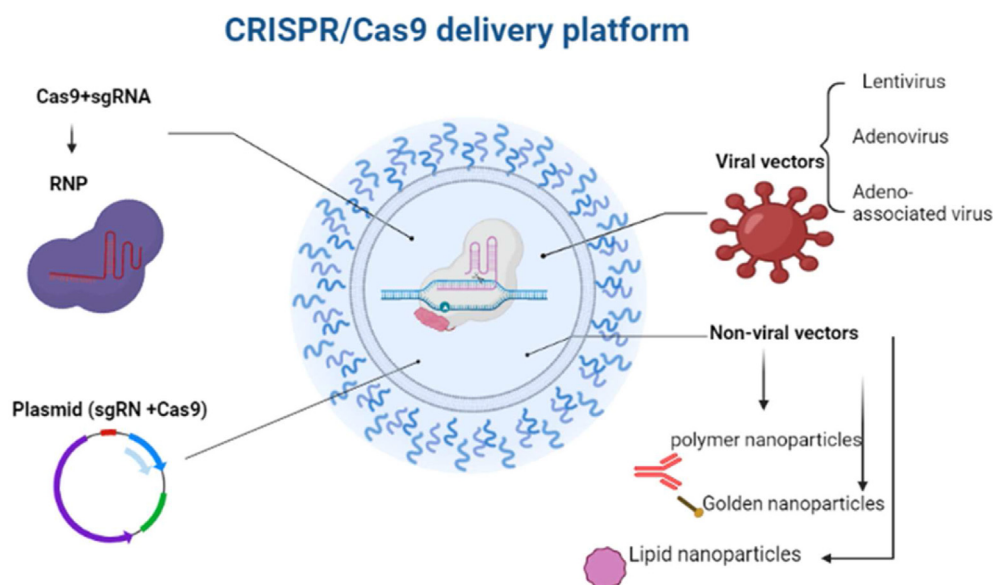
Viral vectors

Recombinant viral vectors have been developed by exploiting the capacity of viruses to insert exogenous genetic material into cells in order to transfer beneficial properties into infected cells/tissues (Table 2).[70] Among several viral vectors, adeno-associated viruses (AAV), lentivirus, and adenovirus play an important role in genome editing therapies and have been widely used in clinical models and trials. Although viral vectors do not cause severe human disease, they can induce immune responses, which may reduce transfection efficiency.[71] Another problem with viral vectors is the capacity to permanently restore DNA in the host genome, which may lead to off-target effects and translocations.[72]

Adenovirus

Adenovirus is a double-stranded DNA virus with 80–100 nm dimensions. Its genome is about 34–43 kb in length and can store about 8 kb of exogenous DNA in itself.[73] Due to its extraordinary capacity to carry large heritable cargos, the transfection ability of the adenovirus vector-mediated CRISPR/Cas9 module can be enhanced by providing additional targeting signals.[74] Nowadays some adenoviral vectors can accumulate the target DNA up to 37 kb by reducing their primary genomic material.[75] Adenovirus can infect both dividing and non-dividing cells, but an important issue is that its genome is not coordinated with cells, which reduces off-target effects and non-intended mutations.[76] Regarding pathogenicity, the adenovirus vectors can stimulate the immune response,[77] even though this reaction may promote the cytotoxicity effect on tumor cells, the neutralizing agent reaction induced by B cell action is not related to subsequent vector transfer.[78] Therefore, reducing the immune response promoted by adenoviral vector will significantly increase the safety and effectiveness of the transfer by this vector. The use of poly (lactic acid/glycolic acid) copolymer for encapsulation of recombinant adenovirus vectors reduces the immunogenicity of adenoviruses and enhances near-neutralizing antibodies in vitro. This information will be used to improve more advanced viral vectors.[79]

Genetically engineered mouse models (GEMMs) of human cancer are imperative instruments to analyze the atomic components of tumorigenesis.[80] Transferring CRISPR/Cas9 into cells by physical methods utilizing adenovirus vectors actuates particular chromosomal improvements to produce a mouse model of Eml4-Alk-driven lung cancer.[81] This methodology extends how researchers

CRISPR/Cas9 delivery platform

Figure 2 CRISPR/Cas9 delivery platforms showing viral and non-viral vectors, plasmid, and RNP.

Table 2 Viral vectors for CRISPR/Cas9 delivery system.

Delivery	Packaging capacity	Advantages	Disadvantages
Lentivirus	Approximately 10 kb	High transduction efficiency; large cargo size; low immunogenicity; ability to transduce dividing and non-dividing cells in different tissues	Non-specific DNA integration causes cancer risk; complex packaging structure
Adenovirus	Approximately 8—10 kb	Efficient delivery; large cargo size	Inflammatory response
Adeno-associated virus	Approximately 4.7 kb	Multiple serotypes; low immunogenicity; ability to transduce dividing and non-dividing cells in different tissues	Pre-existing neutralizing antibodies; long-term expression of Cas9 causes off-target effects

develop human cancer models by rearranging complex and time-consuming hereditary genomic controls. Thus, adenoviral vectors have been utilized to improve gene editing techniques focusing on Pten in a mouse with nonalcoholic steatohepatitis (NASH). In this model mice infused with an adenoviral vector showed signs of hepatomegaly and NASH after 4 months. Indeed, the signs of typical adenoviral vector-related immunotoxicity in the liver appeared while adenoviral vectors intervened in effective Pten gene editing, giving a novel strategy to imitate human liver malady in mice.[82] GEMMs produced by site-specific recombinase innovation are non-efficient and time-consuming, but adenoviral vector-mediated CRISPR/Cas9 gene editing can successfully deliver numerous subtypes of delicate tissue sarcoma in wild-type mice and GEMMs. Whole-exome sequencing validated that sarcomas produced utilizing CRISPR/Cas9 are comparative to those produced utilizing conventional recombinase innovation, demonstrating the system's potential to quickly create cancers with comparative genotypes and phenotypes as conventional techniques.[83]

Adeno-associated viruses (AAVs)

AAVs comprise an icosahedral protein capsid with a breadth of ~26 nm and an ssDNA genome of ~4.7 kb.[84] AAV vectors have interesting properties, such as the need to be virulence (living in other cells in order to be replicated), long-term gene expression, and the capacity to live in dividing and non-dividing cells, so they are widely used for *in vivo* transfection systems.[84,85] In the expansion of the AAV family, AAV vectors are characterized by serotype phenotyping and have variable tropism focusing on different organs.[92,132] Even though AAVs are excellent delivery vehicles, they have some shortcomings in their use to deliver CRISPR/Cas9 *in vivo*. The ideal AAV transmission capacity is estimated to be 4.1—4.9 kb. Even though vectors are larger

than their genome size, packaging efficiency is severely reduced.[86] For example, the SpCas9 protein is ∼4.2 kb, and recombinant AAV must also contain critical regulatory components for gene expression, so AAVs cannot be used to provide extensive gene regulation.[84]

When using AAVs for transfection, SpCas9 and sgRNA must be encoded on different vectors.[58,87] Another important issue of AAV is the presence of pre-existing neutralizing antibodies against AAV in patients with previous AAV infection, which significantly reduces the transfection efficiency.[88] In many cases, the combination of capsid regulation and genome regulation to provide an optimal AAV serotype vector can reduce the avidity of neutralizing antibodies, subsequently reducing resistant reactions and providing stepwise transfection efficiency.[89] Furthermore, long-term expression of the AAV transgene may also be an opportunity, as the continuous expression of the Cas9 core may cause significant off-target effects.[90] There are problems in the mass production of AAVs. Even though there are still several challenges to overcome, scientists have tried to explore AAV-mediated CRISPR transfection systems. The AAV dual-carrier framework effectively targets a single gene or multiple genes in the mouse brain and characterizes the effects of genome regulation on neurons,[91] suggesting that AAV-mediated genome editing could be useful for brain diseases. As distinct AAV serotypes have broad tissue tropism, AAV-mediated genome editing can also be used to generate existing cancer models.[92] Platt et al delivered a single AAV vector into the lungs of mice models to interfere with p53, Lkb1, and KrasG12D genes leading to adenocarcinoma. Furthermore, the use of AAV to deliver sgRNA to Cas9 knockout mice can be used for high-throughput *in vivo* editing to generate mouse models of the desired cancer.[93]

Lentivirus

Lentivirus is a subset of the retrovirus family, and its genome contains a single-stranded RNA of 7—12 kb.[94] Lentiviral vectors achieve successful cell transfection in various cell types (dividing and non-dividing cells) with a reduction of required culture time for cell transfection. Compared to adenovirus or AAV vectors, lentivirus appears to have less cytotoxicity and immunogenicity and fewer adverse effects on transduced cells.[95] Furthermore, lentiviruses are promising as *in vivo* transfection cargo due to their relative ease of use. Regularly, the lentivirus integrates its genome into the host genome, which enhances the time of transgene expression. Continuous expression of Cas9 increases the chance of off-target effects and prevents application in high-precision genome edition.[96] Selective and integrase-deficient lentiviral vectors produced by integrase modification can significantly reduce the risk of unintended mutations.[97] In preclinical studies, it seems that lentiviral delivery of Cas9 and direct RNA targeting of the KRAS gene have inhibited the proliferation of cancer cells.[98] CRISPR/Cas9 viral delivery targeting BCR-ABL essentially inhibits cell growth, myelogenous leukemia cells, and tumorigenesis. Thus, therapies based on editing ABL may offer a potential technique for patients with imatinib-resistant myeloid leukemia.[99] Lentiviruses have been approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA).[100]

Non-viral vectors

Safety issues remain a major bottleneck for the lack of widespread clinical use of viral vectors,[101] safe reactivity,[71] and stable expression.[102] As an alternative, non-viral vectors for cancer therapy have been investigated due to their immunogenicity, high biocompatibility, amazing delivery capability, and large-scale production.[103,104] Nanotechnology-based drug delivery frameworks develop broad applications of CRISPR/Cas9 therapy, advance safety, and provide a practical approach to overcoming the challenges of viral vectors (Table 3).

Lipid nanoparticles (LNPs)

LNPs are amphiphilic vehicles composed of various hydrophobic and hydrophilic moieties, such as cationic or ionized

Table 3 Nanotechnology-based delivery systems for CRISPR/Cas9.

Delivery system	Cargo options	Advantages	Disadvantages
Polymer nanoparticles	RNP plasmid DNA; RNP complex; Cas9 mRNA; sgRNA; donor DNA	High biocompatibility; low immunogenicity; ability to reduce off-target effects; mass production; low cost	Toxicity; limited delivery efficiency
Golden nanoparticles	RNP plasmid DNA; RNP complex; Cas9 mRNA; sgRNA; donor DNA	High biocompatibility; low immunogenicity; ability to reduce off-target effects; mass production; low cost	Limited delivery efficiency
Lipid nanoparticles	RNP plasmid DNA; RNP complex; Cas9 mRNA; sgRNA; donor DNA	High biocompatibility; low immunogenicity; ability to reduce off-target effects; mass production; low cost	Degradation *in vivo*

lipids; neutral lipids such as phospholipids or cholesterol, and polyethylene glycol-lipids.[105] LNPs were developed as carriers for the delivery of an array of particles into cells, particularly in nuclear transports. Since nucleic acids are relatively unstable outside the cell and carry many anions, they cannot easily cross the cell membrane. However, the ionic and polar head in cationic liposomes allows for easy delivery of nucleic acids into cells. Compared with conventional drug delivery, LNPs have interesting features including monitoring the cargo, targeted delivery systems, and reduced toxicity. These features have made LNPs attractive for the delivery of anticancer drugs.[106] Preclinical trials found that LNPs can effectively transfer siRNA or mRNA[107,108]; therefore, LNPs seem to be a safe and suitable transfer method.[109]

Within the past years, numerous preclinical studies about CRISPR/Cas9 delivery using LNPs have been performed. Two fundamental strategies are utilized for LNP delivery of CRISPR/Cas9 components like transferring the Cas9/sgRNA plasmid and transferring the Cas9/sgRNA RNP complex. Cas9 mRNA and sgRNA can be effectively stacked on LNPs and precisely transported to the liver of mice while intervening in the mouse model for transthyretin (Ttr).[110] Despite several advances, routine prediction, and planning of LNP delivery to target tissues other than the liver for precise gene modification remains a challenge. Organ-specific targeting allows nanoparticles to provide gene-editing frameworks for specific organs, which is expected to encourage the development of gene-regulating therapies.[111]

Polymeric nanoparticles

Polymeric materials have long blood circulation, as well as high bioavailability, amazing biocompatibility, and degradability, and thus they are considered powerful delivery tools.[112] However, conventional strategies for sgRNA-Cas9 deliveries are RNPs which are in danger by proteases in cells. The protein core and the permeable polymer shell form a modern nanocapsule that can be programmed to be aberrantly degraded or stable at different pH values. Capsule degradation breaks down the outer shell and allows the core protein to enter the cell to perform its normal functions. Albeit its disadvantages, this strategy leads a set of proteins into cells and opens a new way for sgRNA delivery, Cas9-RNP, and cancer therapy.[113] Furthermore, in 2019 Chen et al synthesized a glutathione cleavable covalent cross-linked polymer coating around the Cas9-RNP complex to create a nanocapsule. This nanocapsule performed the gene edition *in vitro* without any cytotoxicity evidence. Topical organization of the nanocapsules in mice produced effective gene edition capabilities.[114] In further studies, Cas9-RNP was efficiently transfected into HEK293-T cells and colon cancer cells and induced genome modification. Crucially, nanoarray targeting of mutated KRAS in cancer cells can successfully suppress tumor growth and metastasis in tumor-bearing mouse models.[115] Guo et al have knocked down the breast cancer oncogene lipocalin 2 (LCN2) in human TNBC cells through CRISPR-polymeric nanoparticles. LCN2 generally restricted the migration of mesenchymal phenotype of human TNBC cells and reduced their invasiveness.[116] In another study, Zhang et al combined nanotechnology and genomic design to disrupt cyclin-dependent kinase 5 (Cdk 5) and reduce *PD-L1* expression in tumor cells. They significantly inhibited murine melanoma development and TNBC lung metastasis.[117] It seems that polymeric nanoparticles have great prospects and wide potential in CRISPR genome editing as a modern precision medicine for cancer treatment.

Gold nanoparticles (GNPs)

GNPs are another alternative carrier for transferring CRISPR/Cas9. GNPs can be combined with distinctive components such as nucleic acids, lipids, or polymers. They have relative biocompatibility and enter into various sorts of cells.[118,119] Attaching different components of nucleic acids and glycoproteins on the surface of the GNPs makes beneficial applications.[120] The pharmacokinetics of GNPs can be controlled by changing their size, shape, charge, and surface area.[121,122] GNPs/Cas9/sgRNA complex can achieve ~30% transfection efficiency, which provides a modern strategy for genomic research.[123] While HDR-based therapies treat most inherited diseases, developing delivery vehicles that can activate HDR in the body has been challenging. A transfer vehicle composed of DNA-conjugated GNPs and cationic endosomal escape polymers can transfer Cas9-RNP to essential cells and stem cells. This complex, called CRISPR-Gold has actuated HDR in MDX mouse myoblasts with negligible off-target effects.[124] Since safety is the main barrier to the entry of GNPs into the human body, it is important to investigate its interaction inside the cell. The uptake of GNPs by resistant mast cells stimulates the production of pro-inflammatory cytokines, suggesting that GNPs have an immunostimulatory effect.[125] In many cells, the interaction of GNPs with different receptors on the surface of cells and different types of endocytosis depends on the regulatory changes caused by GNPs.[126,127] In addition, due to the specific biophysical properties of metal particles, the charge and electrostatic field on the surface of the particles also fundamentally affect safety issues. Further research is needed to fully characterize the components involved in the interaction of GNPs with the immune system.

Challenges and future opportunities

In the future, the use of base editors and core editors in human experiments will have a significant impact on health issues, because there are many inherited diseases caused by point mutations that can be cured by modern types of genome editors with extraordinary levels of minimal undesired off-targets. Some researchers are trying to expand the potential of base and core editors. A significant increase in the number of clinical trials using the CRISPR technique for genome editing has been seen in recent years. In this part, some challenges related to the CRISPR technique will be discussed. Due to the speed of research, a solution to these problems and complications will be also provided.

Challenges of CRISPR/Cas9

Delivery challenge

Even though CRISPR/Cas9 may be a developed gene editing innovation that has been utilized broadly, it keeps up numerous issues due to the off-target effects, efficiency, and safety challenges. In terms of CRISPR-based gene therapy, the challenges of the *in vivo* transfection methods are highlighted.[27] A complete transfection strategy for CRISPR/cas9 should have high transfection efficiency, an exceptional focus on capacity, and ease of mass production. However, the current methods are still far from reaching these goals.[58] Physical methods of CRISPR/Cas9 transfer are commonly used *in vitro* but are uncommon and non-efficient *in vivo*. Different methods like electroporation, microinjection, ultrasound-driven nanomotors,[128] microfluidic/nanofluidic approaches,[129] and nanocarriers[130] have been widely used. For using CRISPR/Cas9 in human stem cell gene editing, electroporation is still the main choice in several studies.[131] However, several physical approaches can deliver CRISPR/Cas9 *in vivo*. For example, hydrodynamic injection (HDI) was described as a novel approach for CRISPR delivery,[50] but its application is limited because this strategy may cause damage during transfection.

Apart from the physical techniques, scientists are delivering CRISPR/Cas9 using diverse carriers. Along with the types of carriers, the CRISPR/Cas9 transfection strategy may be divided into two types: viral transfection methods and non-viral techniques. Due to the greater transfection efficiency, viral vectors are more common for CRISPR/Cas9 transfection. Adeno-associated virus (AAV) is the most common vector for gene therapy *in vivo* and *in vitro* due to its wide serotype, low immunogenicity, and toxicity,[132] but its small carrying capacity (about 4.5–5 kb) must be improved. Compared to AAV, lentivirus (LV) and adenovirus (AdV) have the better, higher, and stronger capacity and transfer the additional genetic components. Therefore, one of the most important points about AdV is its capacity to transfect a wider range of cells than LV and AAV. In some cases, larger LV and AdV sizes may induce humoral responses and indeed cellular immunity.[123] Other viral vectors have been recently developed and have distinct characteristics. For example, EBV vectors can be more stable in cells,[133] and Sendai virus vectors are competent to infect a wider variety of cells,[134] while baculovirus vectors have a higher transfect capacity.[135] However, these vectors are currently used in ambient conditions, but they can still be used *in vivo* after further optimization.

Concerning non-viral techniques, liposomes are utilized frequently since they have been merchandised broadly. In other examples, some researchers attempted to utilize gold nanoparticles (AuNPs) as the vectors for CRISPR/Cas9 transfer. Other non-viral vectors like Lipofectamine RNAiMAX,[136] PolyJet™ In Vitro DNA Transfection Reagent,[137] and X-tremeGENE HP DNA Transfection Reagent[137] are commercial and appropriate for *in vitro* or *ex vivo* tests. In terms of *in vivo* transfection, thousands of researchers are focusing on finding and synthesizing non-viral vectors with high efficiency and low cytotoxicity. These techniques include conventional nano-sized arrays (such as self-assembled micelles[138] and polyethylene glycol-modified cationic lipid nanoparticles[139] for CRISPR/Cas9 plasmid delivery), DNA nanostructures,[140] and phosphorus nanosheets.[141] For CRISPR/Cas9 transfection, receptor-mediated transfection methods (*e.g.*, folate receptor-targeted liposomes of CRISPR/Cas9 plasmids[142]), cell-penetrating peptides (CPPs)-mediated transfection methods, and KASIM transfection methods (*e.g.*, a hybrid of *K9* protein)[143] are used. Wang developed cationic α-helical polypeptide-based PEGylated nanoparticles to deliver CRISPR/Cas9 plasmids and sgRNA.[144] Also, several delivery methods (*e.g.*, R8-dGR did peptide-modified cationic liposome for delivery of CRISPR/Cas9 plasmids and sgRNA[145] and near-infrared transformation-activated framework for CRISPR/Cas9 delivery[146]) were developed. In a recent study, a four-component formula [DOTAP (1,2-dioleoyl-3-trimethyl-lammoniumpropane)/DOPE/cholesterol/Chol-PEG (cholesterol-polyethylene glycol)] liposome with the highest rate of transfection of Cas9/sgRNA resulted in 39% gene-editing efficiency to knockout GFP reporter.[109]

Still, several inconveniences within the field of non-viral procedures, like transfer obstructions and endosomal escape exist.[123]

In comparison to different vectors, the shapes of cargos moreover play an important role in CRISPR/Cas9 transfer. Cas9 is transferred within the shape of DNA or mRNA with sgRNA and layout sequence together. In the case of gene editing productivity, Yin and his colleagues transferred Cas9 mRNA by lipid nanoparticles whereas transferring sgRNA and making layout arrangement by AAV separately.[147] In addition, Cas9 proteins can also be easily transfected into cells by hybridized strategies. This bypasses the risk of genome integrity and reduces the off-target effect due to the short half-life of the Cas9 protein, which is considered a safer approach for gene therapy.[147] Consequently, the smaller the carrying capacity of transfection vectors is, less immune responses will occur; while there is a need for greater capacity to carry more CRISPR. However, multi-functional chimeric peptides due to their size flexibility, biocompatibility, and biodegradability have shown potential abilities in gene delivery that may be promising for CRISPR-based gene editing.[148,149]

Off-target effect

The off-target effect is one of the limitations of the CRISPR/Cas9 technique and is considered a critical risk *in vivo* gene therapy. Although several computer programs have optimized the design of sgRNA, its specificity cannot be fully guaranteed. In addition to sgRNA design, *Cas9* protein also plays a vital role. As an example, the design of Cas9, such as protein transfer, may substantially reduce the off-target effect. The optimized *Cas9* protein as well as using the *SpCas9-HF1*[150] and *eSpCas9*[151] are two approaches to reduce the off-target effect. Notably, the off-target effect remains unresolved *in vivo* and is strongly related to the transfer technique.

PAM limitation

As it was reviewed, the PAM sequence is fundamental for CRISPR/Cas9 and is largely dependent on Cas9 specificity. PAM sequence limits the design of sgRNA and reduces the adaptability of CRISPR/Cas9 with other systems. Even though an expanding number of CRISPR sorts are found, more PAM sequences have been discovered. The obligatory PAM addition still influences the design of sgRNA in a few circumstances. Subsequently, how to create a designable PAM is critical to broadening the application of CRISPR/Cas9.

Immune response

Even though there are not numerous reports about the extreme immune responses caused by Cas9, the antibodies against Cas9 have been broadly recognized in human bodies,[152] which recommends the potential hazard of aggravation against CRISPR/Cas9-based gene therapies. Scientists pay more attention to the immunogenicity caused by vectors, particularly viral vectors, since the human body may have been exposed to them before. Collectively, the immune responses caused by CRISPR/Cas9 gene-editing framework are one of the major hazard components within the improvement of CRISPR-based gene therapy *in vivo*.

Multiple gene-editing

CRISPR/Cas9 is a proficient gene-editing system that changes one gene locus with a sgRNA at a single time. Subsequently, numerous gene-editing techniques which use CRISPR/Cas9 are dependent on different sgRNAs, which decreases the editing efficiency and increases the transfection problem. Later CRISPR/Cas12a has overcome this challenge, but other issues are still unsolved, including the cell inactivation and cell cycle capture after different gene-editing. In the future, convincing different gene-editing techniques will significantly enhance the gene therapy of polygenic maladies and cancers.

Future perspectives

CRISPR/Cas9 as a microbial intrinsic immunity framework has been developed as a robust gene-editing technology. Due to its precision and efficiency, CRISPR/Cas9 techniques can provide an incredible chance to treat a few gene-related diseases by deletion, insertion, regulation, and blocking different genes. Cas9-mediated gene therapy has been utilized to treat different non-cancerous maladies. Monogenetic diseases and X-linked illnesses caused by gene transfection are the most studied diseases by this technique which have even entered clinical trials. In this regard, the probability of both CVDs and NDDs has become lower by using CRISPR/Cas9. At the same time, the treatment of visual illnesses by Cas9 has developed into clinical stages.

CRISPR/Cas9 in the first attempt was used for cancer therapy. Combined with computational simulation methods, Cas9-based target screening gives an advanced approach to monitoring the cancer stages. Deleting onco-genes or turning on the tumor-silencing genes alone or in combination with each other, are the major approaches to treating cancers and viral disorders. Besides, a few investigations illustrated a few gene regulations, epigenetic factors, and micro-environmental genes which play crucial roles in cancer therapy. In some studies, CAR-T cells have been generated by the Cas9 system and reached successfully to clinical trial phases. Thus, CRISPR/Cas9 has shown up as a vigorous gene-editing tool. In some cases, a few issues keep unsolved like off-target effects, transfer challenges, PAM confinement, and immunogenicity. During gene therapy, the off-target effect and editing effectiveness are two of the most concerning issues since the off-target may cause unintended editions in ordinary genes and then lead to some illnesses. Also, the editing efficiency specifically influences the final impact. The transfer of CRISPR/Cas9 is crucial for CRISPR-based treatments. The transfection efficiency determines the proficiency of Cas9-mediated gene therapy to a significant degree. The targetability and transfer time are two factors related to the off-target effect.

As discussed before, expendable plans (such as transferring the Cas9 protein) and all-in-one plans (such as transferring the Cas9 plasmids and sgRNA at the same time) are two ways to reduce the off-target effect. Furthermore, re-engineered or optimized Cas9 proteins decrease the off-target effect as well. Compared with conventional CRISPR/Cas9-based gene editing tools, base edition and primary edition tools do not make DSBs when altering genes. This phenomenon will lead to fewer off-targets. Also, their edition efficiency will be improved using optimizing chemicals or prime editing guide RNA (pegRNA), recommending the awesome potential for clinical application. sgRNA design is still fundamental since it plays a key role in gene targeting. For optimizing sgRNA design, some rules and computer programs calculating some factors like the distance from the PAM sequence may be effective on sgRNA design. As studied, most clinical trials study gene editing in patient-derived cells *ex vivo*, such as the treatment of SCDs. This strategy reduces the hazard of off-target effect and transfer challenges but is not reasonable for all illnesses. The clinical application of CRISPR/Cas9 is still at an early stage, and off-target effects and transfer mechanisms remain two important challenges of Cas9 -based editing tools.

Compared with monogenetic illnesses, cancer therapy by Cas9-based systems is more challenging due to numerous gene mutations in cancerous cells, even though it is possible to apply numerous gene editing by CRISPR/Cas9. CRISPR/Cas9-mediated numerous gene editing technology is not broadly used in clinical applications. It is thought that it has a few potential issues, such as extreme off-target effects and the deletion of enormous DNA fragments.[153] In this manner, novel approaches in multiple gene editing were developed to overcome these challenges. But for CRISPR/Cas9, other CRISPR frameworks, including Cas12a, Cas3 (with Cascade), Cas13, dCas9, and nCas9 are under attention.[154]

In addition, compared to Cas9, Cas12a has a site for the period II CRISPR framework. But Cas12a creates an amazing cut in comparison to Cas9, which could be an incredible

advantage when joining DNA groupings. Within the Cas3 framework, the Cascade complex ties and recognizes the target DNA arrangement at the Cas3 recognition site in order to produce a single-strand DNA sequence. Based on the PAM sequence, Cas3 is more adaptable to target DNA sequences than Cas9. Different from Cas9, Cas12a, and Cas3, Cas13 is an RNA-guided that can alter single-strand RNA proficiently. Both dCas9 and nCas9 lose the nuclease action but keep up the capacity to target DNA groupings, so a part of re-engineered CRISPR/Cas9 devices, such as CRISPRi, CRIPARa, gene editing tools, preliminary editing techniques, *etc*., are based on dCas9 or nCas9. In conclusion, CRISPR/Cas9 is an efficient gene-editing tool but not an idealized therapy approach. CRISPR needs a better safety level. Cell treatment by Cas9 appears to be easier to design whereas it has a few inconveniences during *in vivo* gene therapy approaches. It is noteworthy that only hundreds of diseases can be treated by cell therapy, while a broader range of hereditary maladies can be cured by gene therapy tools like Cas9 which is one of the major viewpoints to be more developed in the future. Effectively and safely gene editing by CRISPR/Cas9 *in vivo* will be done with proper transfection systems within the following decade.

Conflict of interests

The authors declare no conflict of interests.

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