Received: 5 March 2019 | Revised: 25 May 2019 | Accepted: 11 June 2019

DOI: 10.1002/jgm.3107

REVIEW ARTICLE

WILEY

Delivery of CRISPR/Cas9 for therapeutic genome editing

Xiaojie Xu[1] | Tao Wan[1] | Huhu Xin[1] | Da Li[2] | Hongming Pan[2] | Jun Wu[3] | Yuan Ping[1]

[1] College of Pharmaceutical Sciences, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310058, China

[2] Department of Medical Oncology, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, China

[3] School of Biomedical Engineering, Sun Yat-sen University, Guangzhou 510006, China

Correspondence
Y. Ping, College of Pharmaceutical Sciences, Zhejiang University, 866 Yuhangtang Road, Hangzhou, 310058, China.
Email: pingy@zju.edu.cn

Funding information
National Science and Technology Major Project of the Ministry of Science and Technology of China, Grant/Award Number: 2018ZX10301402; Fundamental Research Funds for the Central Universities, Grant/Award Number: 2018XZZX001-14; National Natural Science Foundation of China, Grant/Award Number: 81872807

Abstract

The clustered, regularly-interspaced, short palindromic repeat (CRISPR)-associated nuclease 9 (CRISPR/Cas9) is emerging as a promising genome-editing tool for treating diseases in a precise way, and has been applied to a wide range of research in the areas of biology, genetics, and medicine. Delivery of therapeutic genome-editing agents provides a promising platform for the treatment of genetic disorders. Although viral vectors are widely used to deliver CRISPR/Cas9 elements with high efficiency, they suffer from several drawbacks, such as mutagenesis, immunogenicity, and off-target effects. Recently, non-viral vectors have emerged as another class of delivery carriers in terms of their safety, simplicity, and flexibility. In this review, we discuss the modes of CRISPR/Cas9 delivery, the barriers to the delivery process and the application of CRISPR/Cas9 system for the treatment of genetic disorders. We also highlight several representative types of non-viral vectors, including polymers, liposomes, cell-penetrating peptides, and other synthetic vectors, for the therapeutic delivery of CRISPR/Cas9 system. The applications of CRISPR/Cas9 in treating genetic disorders mediated by the non-viral vectors are also discussed.

KEYWORDS

drug delivery, gene editing, gene therapy, nanomedicine, non-viral vector

1 | INTRODUCTION

The clustered, regularly-interspaced, short palindromic repeats (CRISPR) were originally discovered by Ishino *et al.*[1] in 1987, and CRISPR were found as repeat sequences that were interspaced by nucleotide spacers in the *Escherichia coli* genome. Subsequently, the CRISPR-associated (Cas) genes encoding proteins were observed.[2-5] CRISPR that appeared in bacteria and archaea widely protect themselves from attack by invasive mobile genetic elements, such as plasmids and phages.[6-9] The immune defense functions utilised include three stages. The first stage is the insertion of spacer sequences of invasive nucleic acids into the CRISPR array, and the second stage is the generation of mature CRISPR RNA (crRNA), composed of repeated sequences and spacer sequences that have the ability to target foreign nucleic acids. The third stage is the disruption of invasive nucleic acids via Cas proteins with the guidance of crRNA that is partially complementary to the target site.[10-13]

CRISPR/Cas systems are divided into two classes, which are further subdivided into six types.[14] Classes 1 and 2 include multi-subunit effector complexes and single protein effectors, respectively.[15] CRISPR/Cas9 belongs to the class 2 type II system in prokaryotes, and it was first reported regarding its capacity for editing the mammalian genome by Cong *et al.*[16] To date, the CRISPR/Cas9 system from *Streptococcus pyogenes* Cas9 (SpCas9) has played an important role in genome editing in a wide range of biomedical applications. Compared with early gene-editing technologies, such as ZFNs (i.e. zinc finger nucleases) and TALENs (i.e. transcription activator-like effector nucleases), the CRISPR/Cas9 system exhibits the advantages of simplicity, flexibility, low cost, high specificity, and efficiency.[17-24]

As a widely used tool of genome editing, the CRISPR/Cas9 system consists of three main components: (i) Cas9, which is an RNA-guided endonuclease that can produce blunt-end cleavage in the double-strand DNA at specific sites in the genome. Cas9 contains the HNH

Xiaojie Xu and Tao Wan contributed equally to this work.

nuclease domain, and the RuvC nuclease domain; the former can cleave the target DNA strand that is complementary to single guide RNA (sgRNA), whereas the later cleaves the non-target strand. With dual cleavage effects, Cas9 can generate blunt-ended double-strand breaks (DSBs) in the target DNA.[25-27] (ii) crRNA, which contains the sequences that are complementary to the target DNA sequence in the desired genome loci via Watson–Crick base pairing rules. It is a critical element for the precise direction of Cas9. (iii) Trans-activating crRNA (tracrRNA), which contains a non-variable sequence that hybridizes with crRNA. Generally, the hybrids of crRNA and tracrRNA can create chimeric sgRNA. As a result, the combination of sgRNA and Cas9 nucleases forms ribonucleoproteins (RNPs) that have the ability to mediate precise site-specific DSBs.[26] In addition to the above components, a few bases present downstream of the targeted DNA site are also crucial. These bases, termed the protospacer adjacent motif (PAM), are a critical requirement for Cas9 to identify RNA-guided targeted genome loci.[14] The PAM sequence varies in different types and variants of CRISPR/Cas systems. For example, SpCas9 and *Staphylococcus aureus* Cas9 (SaCas9) require the sequence of 5'-NGG-3′ and 5'-NNGRRT-3′ (R = G or A), respectively.[25,28-30] Collectively, the CRISPR/Cas9 system plays an important role in regulating genomic functions in terms of insertion, deletion, activation, and suppression, etc. These functions repurpose CRISPR/Cas9 for a variety of biomedical applications, including transcriptional control, epigenetic modification, genome-wide screening, and chromosomal imaging. Furthermore, therapeutic genome editing with respect to various genetic disorders have also been extensively investigated in recent years.

CRISPR/Cas9-induced site-specific cleavage results in the activation of inherent DNA repair pathways of non-homologous end-joining (NHEJ) and homology-directed repair (HDR).[26,27,31,32] Additionally, microhomology-mediated end-joining and homology-mediated end-joining also play a role in the repairment process. NHEJ can induce different lengths of insertions/deletions (indels) randomly at the target site. These indels can disrupt the translational reading frame of a coding sequence and result in gene mutation, or knockout. By contrast, exogenous DNA templates are required to repair the DSBs in the pathway of HDR. The sequence of DNA templates can replace the native sequence to correct the deleterious mutation of genes.[33-38] NHEJ occurs more frequently in the process of repairment, which is mainly related to the repetitive nature of the human genome. The endogenous repair pathways are also highly related to the cell state and the presence of donor DNA templates.

Based on the CRISPR/Cas9 system, some other CRISPR technologies have been developed to modulate gene expression. For example, CRISPR-based interference relies on the mechanism of hindering the combination of RNA polymerase with DNA, and is attributed to the steric hindrance of nuclease-deactivated Cas9 (dCas9) binding DNA securely. Therefore, CRISPR-based interference was used to hamper and suppress RNA transcription to further modulate the protein production.[39,40] Furthermore, CRISPR-Cas13a is known as an RNA-guided RNA-targeting technology for mediating RNA binding and knockdown. This platform is more suitable for therapeutic applications than RNA interference, and shows less risk than DNA editing due to its reversibility.[41,42] In addition, Konermann *et al*.[43] recently demonstrated engineered CRISPR effectors of CRISPR-Cas13d from *Ruminococcus flavefaciens XPD3002*, termed CasRx, which showed merit in terms of significant efficiency, accuracy, and specificity. Compared to RNA interference, CasRx has the distinct advantage of low off-target effects, suggesting its enormous potential for treating genetic diseases caused by RNA disorders.

Despite such potential prospects, the prerequisite for effective genome editing is the desired delivery efficiency in cells, tissues, and organs. To date, the delivery of Cas9 and sgRNA remains challenging as a result of the large size of Cas9. Note that the molecular weight of Cas9 endonuclease is about 160 kDa, with an approximate hydrodynamic diameter of 7 nm.[44,45] Moreover, the sgRNA bears a negative charge that is related to rich PO^{3-} groups of the bases. Thus, developing vehicles that can package these elements for intracellular delivery is critical for achieving desirable gene-editing effects. In general, the delivery system for plasmid encoding Cas9 and sgRNA can be broadly divided into two categories. The first comprises of viral vectors, including adenovirus, adeno-associated virus (AAVs), lentivirus, etc.[46-49] The second comprises of non-viral vectors, including physical and chemical methods. So far, viral vectors have been widely used to deliver CRISPR/Cas9 elements for the treatment of genetic diseases.[50-55] For example, Duchenne muscular dystrophy (DMD) is a fetal disease with the symptoms of cardiomyopathy and heart failure. Long *et al*.[56] investigated viral delivery approach to treat DMD via the disruption of gene mutations in the dystrophin using the neonatal *mdx* mice model. In their study, *mdx* mice were treated by AAV9 that contained the gene-editing plasmid encoding Cas9 and sgRNA. The results showed that this approach restored the expression of functional dystrophin, and further suggested the ability of monogenic disease therapy. However, most viral vectors pose the risk of integration into the host genome. Furthermore, concerns about clinical safety derived from the continuous expression of foreign Cas9 by viral delivery are also emerging, including tumorigenesis, immunogenicity, and off-target effects.[57-59] Viruses also suffer from limited package capacity, which severely hampers the delivery of SpCas9 plasmids longer than 4 kb. For example, as a representative type of viral vector, the package capacity of AAVs is constrained to approximately 4.7 kb. Consequently, most of the viral vectors are incapable of loading the CRISPR/Cas9 cargo completely. Physical methods mainly include electroporation, hydrodynamic injection, and microinjection. Such methods can generate transient holes on the cell membrane to enable direct access for Cas9 intracellular delivery. In particular, physical methods can bypass the obstacles in most of the delivery strategies.[60-65] In an earlier study, Kim *et al*.[66] delivered RNPs instead of plasmids to modify genes in cells via the pathway of electroporation, and revealed that high gene-editing efficiency could be achieved in human primary and embryonic stem cells that were hard to transfect. Nevertheless, physical methods are largely used *in vitro*, but have the limits with respect to *in vivo* applications, primarily as a result of various physiological obstacles. As an alternative, chemical delivery methods, which display the merits of favorable biocompatibility, low immunogenicity, and flexible cargo size, have received more attention

recently. Additionally, they have a reduced risk of immune responses and are free of any risk of insertion into the host genome. At present, a few types of delivery vectors have been exploited for the delivery of genome-editing agents, such as liposomes, polymers, cell-penetrating peptides (CPPs), DNA nanostructures, etc.[67,68]

In this review, we elaborate first on the modes of CRISPR/Cas9 delivery and the barriers to this process. Secondly, the application of CRISPR/Cas9 in different areas, such as imaging, treatment of diseases and constructions of animal models, is introduced. Finally, we discuss the non-viral vectors of the CRISPR/Cas9 system and its application in different therapies of diseases.

2 | FORMAT OF CRISPR/CAS9 DELIVERY

The delivery of CRISPR/Cas9 systems mainly rely on three formats. The first format is the delivery of plasmid DNA, which encodes Cas9 and sgRNA. The second format is delivering the elements of mRNA and sgRNA, with mRNA being converted into Cas9 nucleases via the process of translation in the cytoplasm. The last format of CRISPR/Cas9 delivery is RNP, the Cas9 protein and sgRNA complex that shows the advantages of its safety and low off-target effects.[69] The Cas9 nucleases generated by plasmid DNA are relatively stable and of low cost; however, plasmid DNA also has the potential to express the CRISPR/Cas9 elements persistently, which greatly enhances the potential to generate off-target effects at undesired genome sites and sometimes even causes genetic mutations. By contrast, the delivery of mRNA and proteins represents a safe mode and shows strong activity to modify genes to sufficiently generate gene-editing effects; yet their relatively short half-life well circumvents the limitations of plasmid DNA. Here, we review the modes of CRISPR/Cas9 delivery, and further summarize and compare both its merits and drawbacks (Figure 1).[70-72]

The first format of delivery is the plasmid. After the plasmid enters the nucleus, it expresses the corresponding components of the proteins produced by the following processes of transcription and translation. Compared with the delivery of mRNA and proteins, the transportation of plasmids into the nucleus is a prerequisite to expressing the elements of CRISPR/Cas9. In general, the overall expression cassette of SpCas9 is typically more than 4 kb and, if the plasmid is inserted with reporters, the size will increase. Thereby, many available vectors show difficulties with respect to packing plasmids of such a large size, as well as mediating its efficient delivery.[47,63,70,73] Furthermore, the delivery of the plasmid also faces the risk of random insertion into the host genome, due to the extended persistence in cells, and suffers from higher off-target effects than mRNA and proteins, as a result of the stable and prolonged expression of Cas9.[74,75] Moreover, the cells would activate cyclic GMP-AMP synthase and cause host immunogenicity in response to the transfection of plasmid DNA (Figure 2).[76-78]

The second delivery format is via RNA. mRNA are known to induce transient expression to avoid insertional mutagenesis.[79,80] Compared with plasmid RNA, the intracellular delivery process of mRNA is easier because the process of translation is in the cytoplasm instead of the nucleus. In some cases, delivery vectors are required to load mRNA and sgRNA simultaneously. Because mRNA are labile and vulnerable, the delivery of mRNA suffers from degradation in the process of operation, formulation, and delivery (Figure 3).

The last delivery option is via RNPs. The delivery of RNPs can circumvent the processes of transcription and translation, which

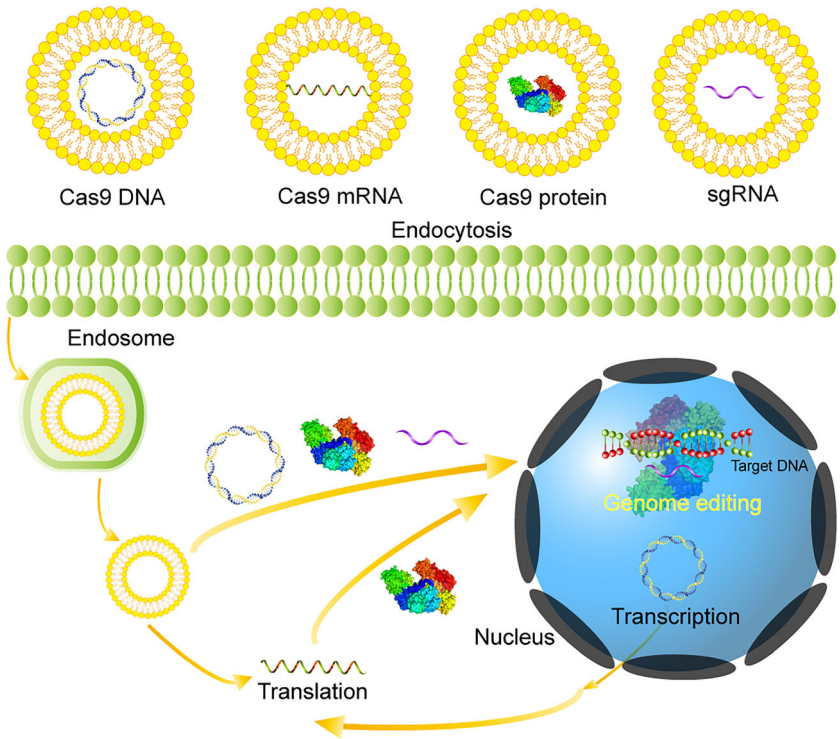

FIGURE 1 Schematic illustration of different configurations of Cas9/gRNA elements and intracellular delivery mediated by the non-viral vectors. For *in vitro* and *in vivo* genome editing, there are typically three formats of Cas9 and sgRNA delivery, namely plasmid, mRNA, and ribonucleoprotein. The first is for delivering plasmid DNA encoding Cas9 proteins and sgRNA. Such plasmids can be transcribed and translated into Cas9 proteins and sgRNA after intracellular delivery. The second option is to deliver messenger RNA (mRNA) and sgRNA. The delivered mRNA can be converted into Cas9 nucleases via the process of translation in cytoplasm. The last form of CRISPR/Cas9 delivery is Cas9 protein complexed with sgRNA, producing ribonucleoproteins (RNPs), that takes advantage of its safety and low off-target effects

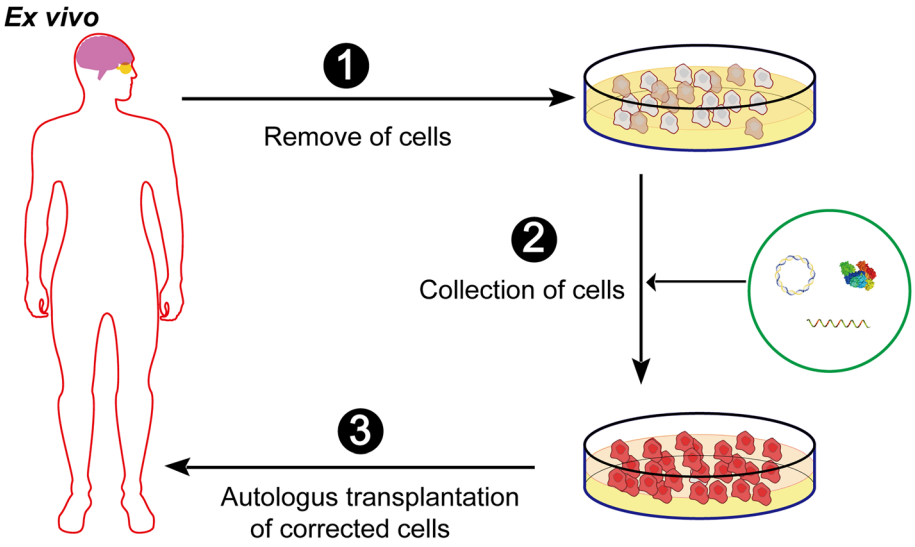

FIGURE 2 Factors including cell isolation, transfection, and transplantation influencing the therapeutic efficacy for genome editing *ex vivo*. The edited cells are shown in red and unedited cells are shown in pink

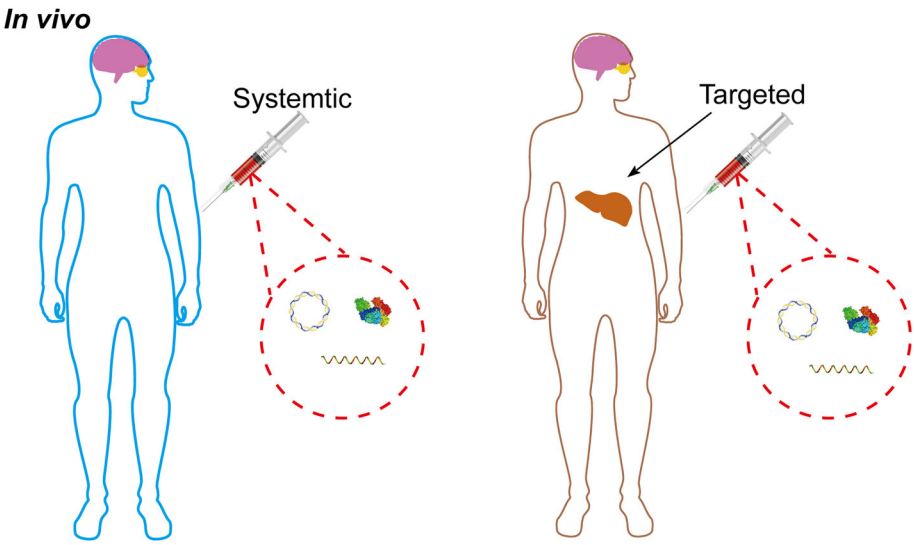

FIGURE 3 Factors influencing the therapeutic efficacy for genome editing *in vivo*. *In vivo* editing therapy is mainly divided into systemic treatments and targeted treatments. Factors such as formulation serum stability, targeted tissue-specific delivery, and the microenvironment of lesions, are the limiting barriers for *in vivo* delivery of CRISPR/Cas9 genome-editing agents

generates the fastest gene-editing effect over the delivery of plasmids and mRNA. Moreover, Cas9 nuclease can be degraded rapidly in cells, suggesting its transient effects in genome editing and a potential role in decreasing off-target effects. Indeed, an earlier study has shown that the delivery of RNPs results in high gene-editing efficiency and low off-target effects.[74] Moreover, some studies indicate that the delivery of RNPs has the advantage of stabilizing sgRNA, partially due to the protective effect of Cas9 proteins to sgRNA.[65] Unlike plasmid DNA, the delivery of RNPs can bypass the risk of integration into genome.[66] Nevertheless, the direct delivery of proteins *in vivo* also exhibits some drawbacks. For example, the Cas9 nucleases serve as inducers to humoral immunity in the body and are cytotoxic towards cells.[81,82]

3 | DELIVERY BARRIERS

The delivery of CRISPR/Cas9 elements to the desired site with the appropriate concentration is a prerequisite for achieving effective gene editing. As an important class of delivery carriers, synthetic nanomaterials play an important role in delivering the elements of CRISPR/Cas9 systems. However, a number of physiological barriers,

from extracellular to intracellular compartments, severely restrict the delivery of CRISPR/Cas9 elements, as discussed below.

3.1 | Extracellular barriers

The first barrier is opsonization, which mediates the clearance of delivery carriers from circulation in the blood. The proteins in serum adhere heavily to the surface of nanocarriers to form corona, and the nanocomplexes are easily internalized by the mononuclear phagocyte system.[83] Additionally, opsonization often shields modified ligands over the carriers from recognizing targeted receptors. Note that the cationic nanoparticles are phagocytosed easier than anionic and neutral nanocarriers.[84] Moreover, studies have indicated that nanoparticles endowed with stealth properties can afford longer circulation in the blood after intravenous injection. For example, polymers modified with polyethylene glycol (PEG) could significantly improve the circulation time in the blood. In addition to modification with stealthy polymers, some other biomimetic components, such as erythrocyte and platelet membranes, show the remarkable ability to resist the process of opsonization.[85-87] Furthermore, after the administration of the nanoparticles, they must contact vascular endothelial cells for extravasation into the desired areas.[88] If the nanoparticles are exploited for tumor targeting, another barrier is the extracellular matrix in the tumor microenvironment. The extracellular matrix is a complex network with physical rigidity, posing a hindrance to the diffusion of nanoparticles into the tumor tissue.[84,89] Although the leaky vasculatures in the tumor regions contribute to the extravasation of nanoparticles, the tumor tissue is also accompanied by fluid extravasation, that leads to an increase of interstitial fluid pressure, which retards the movement of nanoparticles from vasculatures to the tumor microenvironment (Figure 4).[90]

3.2 | Intracellular barriers

The internalization pathways of nanoparticles mainly consist of phagocytosis, micropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin/caveolae-independent endocytosis. For lipid nanoparticles or nanoparticles decorated with lipid membranes, the lipid fusion process plays an important role in cellular uptake. The efficiency of intracellular delivery is impacted by the properties of the nanoparticles, such as size and surface charge.[91,92] To date, many strategies have been implemented aiming to optimize the process of cellular uptake, such as size change, charge reversal, or ligand conjugation.[93-95] After internalization, most nanoparticles enter endosomes. Both the delivery vector and its cargo, especially biomacromolecules, could potentially be degraded by the harsh environments within the endosomes, as a result of the presence of rich enzymes and their acidic conditions. Thus, it is critical to accelerate endosomal escape and facilitate cytosolic and nuclear delivery. Endosomal escape could be achieved either by disrupting the membrane directly, or by bypassing the endo-/lysosome.[96,97] For example, the polymeric carrier, polyethylenimine, could lead to the fragmentation of endosome membranes via the proton-sponge effect.[98,99] After the cargois released into cytoplasm successfully, the CRISPR/Cas9 elements must enter the nucleus for editing purposes. Nuclear entry may be alleviated by modifying either the cargo or carriers with nuclear localization sequences.[100,101] Furthermore, the condensed cytoplasm contains quite numerous organelles, which impede the movement of nanoparticles to the desired organelle (Figure 4).

4 | THE APPLICATION OF THE CRISPR/CAS9 SYSTEM

The genome engineering technology of CRISPR/Cas9 demonstrates a broad range of applications in biology and medicine, such as imaging, modeling, and therapy. By designing appropriate sgRNA, the CRISPR/Cas9 system can target virtually any desired genome sequence. Additionally, multiplex genome editing is also simultaneously achievable by targeting multiple genome sites with different sgRNAs.[102,103] In terms of the construction of animal models, the

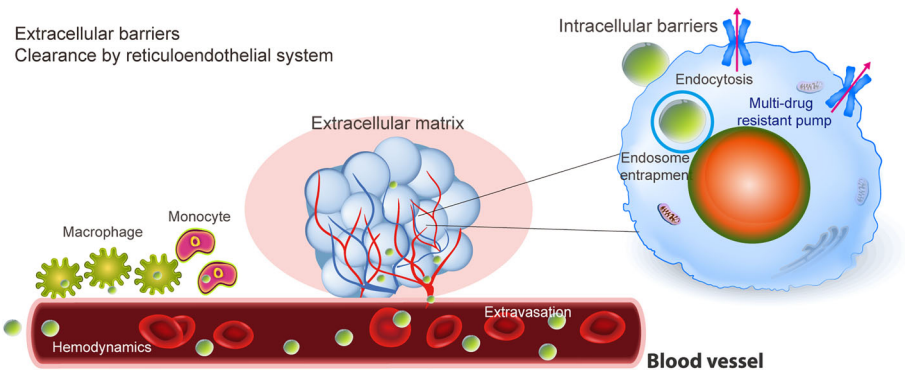

FIGURE 4 Physiological barriers for nanocarrier-mediated drug delivery. After intravenous injection, nanocarriers undergo the process of opsonization, which mediates the clearance of nanocarriers in the blood by the reticuloendothelial system. The hemodynamics have an impact on the movement of nanocarriers to contact vascular walls, and the migration is a prerequisite for extravasation. After the nanocarriers extravasate to the tumor microenvironment, the compact extracellular matrix poses another barrier to the diffusion of nanocarriers to tumor cells. The nanocarriers are internalized by endocytosis, followed by the formation of endosomes. Endosomal escape is necessary for the cargo to reach the targeted organelle

CRISPR/Cas9 system represents a new platform that simplifies the process and is less time-consuming, as opposed to traditional methods. The animal models established by CRISPR/Cas9 not only reveal the phenotype of diseases, but also recapitulate genetic mutations in patients. It has been reported that CRISPR/Cas9 could introduce multiple mutations in human colonic epithelium cells to obtain mutant organoids in a niche-based selection system.[104] In addition to the construction of models, the CRISPR/Cas9 system has tremendous potential in the treatment of diseases, such as monogenic disorders, non-monogenetic diseases, and infectious diseases.[105-107]

4.1 | Imaging

The dCas9, which is short of endonuclease activity to cut double-strand DNA, preserves the inherent capability to combine DNA in the specific genomic site under the guidance of sgRNA.[108] By tethering fluorophores to dCas9 or sgRNA, this system exhibits extensive potential for genome imaging at certain loci within living cells and offers the advantage of capturing live processes of nucleic events. However, traditional imaging techniques, such as fluorescence *in situ* hybridization, are limited in the dynamic imaging as a result of the demand of sample fixation during the process of DNA labeling.[109] By taking advantage of the features of dCas9/sgRNA, a series of elegant methods have been designed for imaging nucleic events. For example, Chen *et al*.[109] first designed a powerful tool that relies on orthogonal dCas9 proteins to label specific DNA loci for imaging in living cells. Later, Wu *et al*.[110] presented a nuclear imaging probe that is composed of a molecular beacon, sgRNA and dCas9 for monitoring chromatin dynamics.

4.2 | Modeling

In sharp contrast to the traditional methods for generating transgenic models in a time-consuming manner, the CRISPR/Cas9 system has demonstrated its superiority in a shorter time span, as well as the closer simulation of human diseases at both phenotype and genetic levels.[111] Furthermore, the multiplex editing capability of CRISPR/Cas9 enables the possibility of establishing polygenic disease models. For example, the progression of cancer is related to the multiple mutations of tumor suppressor genes and oncogenes. By using CRISPR/Cas9 technology, many kinds of cancer models have been established, such as hepatoma, lung carcinoma, and colorectal cancer, etc.[53,104,112]

4.3 | Therapy

Subsequent to understanding the genetic mutations in patient populations, the development of new strategies for treating disorders at the genetic level, instead of the disease phenotype, is imperative.[113] SpCas9 has been widely used in various cell types and species, such as the roundworm, zebrafish, mouse, fruit fly, pig, and monkey, etc.[111] CRISPR/Cas9 is also a promising approach for investigating typical polygenic human diseases, such as diabetes, schizophrenia, and heart disease. Due to its convenience and low cost, CRISPR/Cas9-based genome-editing technology has been extensively studied as a therapeutic tool in many laboratories worldwide in an attempt to accelerate the development of new personalized medicine for treating rare genetic variants. In addition to the promising treatment of inherited disorders, Cas9-mediated genome editing also has the potential to combat non-genetic diseases, and it has been reported that its restraint of the CCR5 receptor in lymphocytes via the NHEJ pathway could circumvent HIV infection.[114] Similarly, the CRISPR/Cas9 system also shows the potential to address the diseases of hypercholesterolemia and hyperlipidemia by deletion of PCSK9 and angiopoietin, respectively.[115,116]

5 | *EX VIVO* VERSUS *IN VIVO* DELIVERY

For therapeutic genome editing *in vivo*, there are generally two routes for the delivery of CRISPR/Cas9. One is *in vivo* delivery, where the genome-editing agents are directly injected into the body via certain administration routes for direct transfection of such components. The other is *ex vivo* delivery, where the cells were first isolated from the animal or human body, followed by *in vitro* transfection and administration of edited cells back into the body. To date, both viral and non-viral delivery vectors have been adopted for the therapeutic application of genome editing. To be considered as a therapeutic drug, the dosage of CRISPR/Cas9 must achieve the therapeutic window after *in vivo* delivery.[117] Ideally, the delivery of CRISPR/Cas9 should target the lesions of disease, so as to maximize the therapeutic efficacy, as well as minimize the accumulation in non-targeted organs (Figure 3). Although direct *in vivo* administration is straightforward and easy to conduct, various extracellular delivery barriers limit the CRISPR/Cas9 editing efficiency, thereby significantly impairing the endonuclease activity of Cas9. Alternatively, *ex vivo* delivery offers a more efficient approach as a result of editing autologous cells *in vitro*. However, the complicated processes of cell isolation, culture, and transplantation make its wide adoption difficult (Figure 2).[118]

6 | NON-VIRAL VECTORS OF CRISPR-CAS9

Recently, broad interest in developing non-viral vectors of CRISPR/Cas9 elements has catalyzed a number of studies showing the promising prospects of this delivery approach. Non-viral vectors can be obtained by rationally designing the chemical structure of polymeric or inorganic materials. In addition, the optimization of non-viral vectors, such as serum stability or targeting ability, can be achieved by proper chemical modifications.[85] Most biomacromolecules, such as Cas9 proteins and mRNA, must be protected to maintain stability and activity in the body. Chemical vectors usually encapsulate the CRISPR/Cas9 elements by physical forces, so that the cargo can partially avoid extracellular and intracellular degradation. To date, non-viral vectors that can be used to deliver CRISPR/Cas9 elements, such as liposomes, polymers, and

TABLE 1 Examples of non-viral vector-mediated CRISPR/Cas9 delivery for the treatment of genetic disorders.

Disease type	Cells or organism	Delivery mode	CRISPR-Cas9 mode	Reference
Monogenic disorders				
Duchenne muscular dystrophy (DMD)	Mouse muscle tissue	Gold nanoparticle	Cas9 protein and sgRNA	Lee *et al.*[119]
Fragile X syndrome (FXS)	Mouse brain	Gold nanoparticle	Cas9 protein and sgRNA	Lee *et al.*[120]
Non-monogenetic disorders				
Type 2 diabetes (T2D)	Macrophages & Monocytes	Polymer	Cas9 mRNA and sgRNA Plasmid DNA encoding Cas9 and sgRNA	Xu *et al.*[121] Luo *et al.*[122]
Monosodium urate crystal (MSU)	Macrophages	Polymer	Cas9 mRNA and sgRNA	Xu *et al.*[121]
Septic shock	Macrophages	Polymer	Cas9 mRNA and sgRNA	Xu *et al.*[121]
Ovarian cancer	SKOV3	Polymer	Plasmid DNA encoding Cas9 and sgRNA	Li *et al.*[123]
Melanoma	A375 cells	Liposome	Cas9 protein and sgRNA Plasmid DNA encoding Cas9 and sgRNA	Wang *et al.*[124] Zhang *et al.*[125]
Malignant glioma	U87 cells	Hydrogel	Cas9 protein and minicircle DNA	Wan *et al.*[69]
Cervical cancer	Hela cells	Polymer	Plasmid DNA encoding Cas9 and sgRNA	Lao *et al.*[126]

CPPs, have been reported for a several genetic disorders. These vectors could interact with cargo to form nanoparticles that have significant advantages with respect to cargo incorporation and targeting specificity. Below, we discuss the application of non-viral vectors for genome editing in more detail (Table 1).

6.1 | Polymers

The inherent flexibility of the polymer structure makes it a fascinating class of delivery material for CRISPR/Cas9 elements. By the rational design of a chemical structure, polymeric carriers can bypass the issues of serum instability in contrast to lipid carriers.[98,127] As non-viral vectors, polymers have been studied intensively for delivering CRISPR/Cas9 elements, largely as a result of their biocompatibility, flexibility, and simplicity, etc.[128,129]

6.1.1 | Polymer-mediated Cas9 plasmid delivery

Ovarian cancer is a lethal disease that poses serious threats to women. Ovarian cancer cells primarily disseminate into the ascites of the peritoneal cavity, followed by metastasis to other organs. To treat ovarian cancer, Li *et al.*[122] reported a multifunctional nucleus-targeting artificial virus (RRPHC) for delivering a CRISPR-Cas9 system. The vectors possessed the core-shell structure, where the complex of fluorinated polymer (PF33) and CRISPR/Cas9 plasmids (Cas9-hMTH1) served as the core. The multifunctional shell (RGD-R8-PEG-HA, RRPH) was coated on the surface of the core to obtain RRPHC. RRPHC contained the mixed components of hyaluronan (HA) and R8-RGD tandem peptide, which endow the merits of intracellular penetrating ability and the targeting function of the nanocarrier. CRISPR/Cas9 plasmid was delivered by RRPHC to knock down the expression of the MutT homolog1 (MTH1), which resulted in more oxidized dNTP accumulation in the ovarian cancer peritoneal metastasis model to induce tumor apoptosis (Figure 5A).[123] In another study, Wang *et al.*[130] developed a versatile gene-editing platform that exhibited a high membrane-penetrating ability for delivering the plasmid of Cas9 and sgRNA for cancer treatment. The vector of PEGylated nanoparticles was constructed with the component of helical polypeptide poly(γ-4-((2-(piperidin-1-*yl*) ethyl) amino-methyl) benzyl-L-glutamate). The nanoparticles showed a perfect deletion efficiency of polo-like kinase 1 (*Plk1*) in HeLa tumor tissue, which decreased the expression of Plk1 protein by 66.7%, and significantly inhibited tumor growth, thereby prolonging the survival time of tumor-bearing mice (Figure 5B).[130] Inflammation is an immune response for the process of clearance of foreign invaders, and the generation of the obesity-associated type 2 diabetes (T2D) has a strong relationship with chronic inflammation. Neutrophils serving as immune cells play an important role in accelerating the progression of chronic inflammation. Therefore, the disruption of neutrophil elastase genes, which were generated by the inducement of high-fat-diet (HFD) in T2D mice, can facilitate the remission of insulin resistance. Liu *et al.*[131] demonstrated cationic lipid (BHEM-Chol)-assisted PEG-PLGA nanoparticles (CLANs), based on the library of poly (ethylene glycol)-*b*-poly (lactide-*co*-glycolide) (PEG-b-PLGA) cationic lipids. CLANs could successfully deliver the plasmid encoding Cas9 and sgRNA, and it was confirmed that the platform can repress the expression of inflammatory factors and provide a promising approach for treating diseases of inflammation (Figure 5 C).[131] Another strategy for treating T2D was also demonstrated by the same research group. In this study, cationic lipid-assisted PEG-*b*-PLGA nanoparticles (CLAN) were used to pack the plasmids, pM330 or pM458, that contain the human CD68 promoter to drive gene expression in specific cells (monocytes and macrophages). The mice were treated by intravenous injection of CLANs to disrupt the expression of netrin-1 genes in monocytes and macrophages specifically, and the results obtained suggested that this could reduce macrophage retention and ameliorate the profiles of glucose tolerance and insulin sensitivity to control T2D (Figure 5D).[122]

Recently, Liang *et al.*[132] developed a type of lipopolymer for CRISPR/Cas9 delivery. In this research, CRISPR/Cas9 plasmids encoding Cas9 proteins and sgRNA targeting vascular endothelial

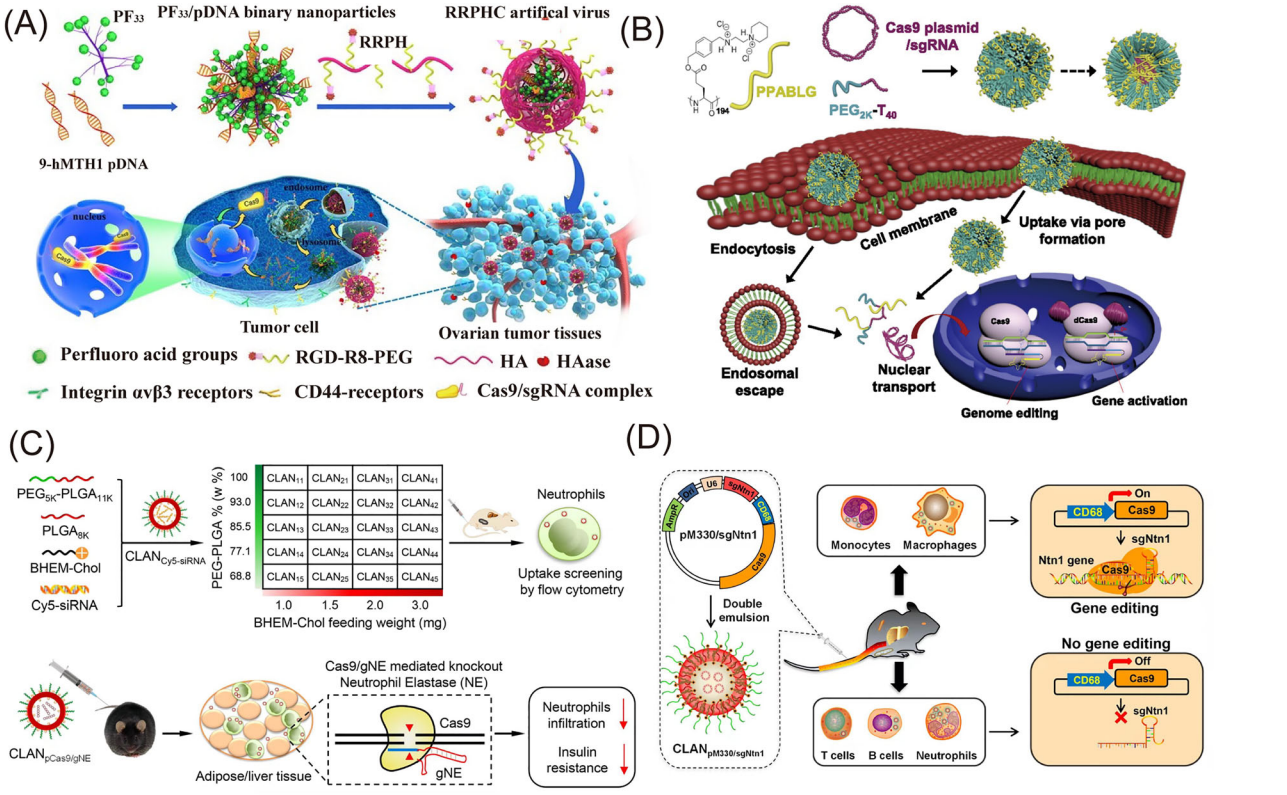

FIGURE 5 Polymer-mediated delivery of Cas9 plasmid for therapeutic genome editing. (A) A multifunctional nucleus-targeting "core-shell" artificial virus (RRPHC) was constructed for the delivery of a CRISPR-Cas9 system that can effectively target ovarian cancer. Reproduced with permission.[123] (B) Cationic *α*-helical polypeptide poly(γ-4-((2-(piperidin-1-yl)ethyl)aminomethyl)benzyl-L-glutamate) (P-HNPs) for the delivery of Cas9 expression plasmids and sgRNA targeting the polo-like kinase 1 (*Plk1*) genes. Reproduced with permission.[130] (C) Screening cationic lipid-assisted nanoparticle (CLAN) to encapsulate pCas9/gNE for the treatment of HFD-induced T2D by knocking out the neutrophil elastase of neutrophils. Reproduced with permission.[131] (D) Macrophage-specific *in vivo* gene editing using cationic lipid-assisted polymeric nanoparticles. Reproduced with permission[122]

growth factor A (VEGFA) were loaded into PEG-polyethylenimine-cholesterol lipopolymers, which were modified with the osteosarcoma (OS) cell-specific aptamer (LC09) to increase OS tumor-selective delivery. VEGFA is highly expressed in OS cells, and the inhibition of VEGFA expression can improve symptoms of poor prognosis, such as orthotopic OS growth and bone lesions.[132]

Polymeric micelles also show a great potential to deliver Cas9 plasmids for gene editing. Recently, self-assembled micelles that are composed of two components were constructed. The first is a poly(propylene oxide) that contains the terminal groups of quaternary ammoniums (PPO-NMe_3) to condense the plasmid. The second is amphiphilic Pluronic F127 that includes the hydrophobic part to interact with PPO-NMe_3, and the Cas9 plasmid was formulated into micelles by hydrophobic and electrostatic self-assembly. This strategy was used to target the HPV18-E7 oncogene associated with human papillomavirus pathogenesis. Proteasome activity can be decreased by 31.9% after transfection for 72 hours, whereas the cells showed 68.1% viability for the Cas9 control.[126] Recently, a facile method to construct a multifunctional vector that can deliver CRISPR/Cas9 plasmids into cancer cells was also developed. The gene-editing tools were delivered into cancer cells for the disruption of *CTNNB1* genes. In detail, the plasmid was first complexed with protamine and then co-precipitated with $CaCO_3$, and the final product was modified with the carboxymethyl chitosan. The carboxymethyl chitosan was further decorated with AS1411 and CPP (TAT), respectively. Among these components, AS1411 offered the targeting property to the cancer cells/nucleus and TAT was used to promote cellular uptake and endosomal escape. By down-regulating β-catenin, the expression of proteins associated with tumor development was also suppressed, such as vimentin, Snail, and MMP-2. This research confirmed that the inhibition of tumor growth, migration, and invasion, as well as cancer stemness, could be attained by non-viral delivery of CRISPR/Cas9.[133]

6.1.2 | Polymer-mediated Cas9 mRNA delivery

NLRP3 inflammasome is a crucial mediator of inflammatory diseases and has been investigated as an effective target for the treatment of multiple inflammatory diseases. Recently, a new strategy that utilized CLAN nanoparticles to deliver the CRISPR/Cas9 system in the form of Cas9 mRNA and NLRP3 sgRNA was reported to alleviate inflammatory disease. Similar to previous efforts, the construction of CLANs is based on PEG-*b*-PLGA polymers and cationic lipids. Because of the cationic nature of BHEM-Chol, CLAN could easily load anionic mRNA

and sgRNA. The CRISPR/Cas9 elements were delivered into macrophages to reduce the generation of NLRP3 inflammasome. The indel frequency of NLRP3 genes in bone marrow derived macrophage can reach 70.2% at 24 hours by treatment of CLANs with a dose of 2.6 nM. To confirm the NLRP3 knockout efficiency *in vivo*, CLANs that were loaded with Cas9 mRNA and NLRP3 sgRNA were injected into C57BL/6 mice. Interestingly, the NLRP3 knockout efficiency in peritoneal macrophages can reach 47.1% at 24 hours with a dose of 2 mg kg^{-1}. Collectively, these results confirmed the effective gene editing of the NLRP3 gene as mediated by CLANs both *in vitro* and *in vivo*. It was further demonstrated that some acute inflammations, such as lipopolysaccharide (LPS)-induced septic shock, monosodium urate crystal (MSU)-induced peritonitis, and HFD-T2D, could be alleviated by disrupting the expression of NLRP3 in macrophages as well (Figure 6).[121]

6.2 | Gold nanoparticles

6.2.1 | Gold nanoparticle-mediated Cas9 ribonucleoprotein delivery

DMD is a congenital and lethal disease that is caused by mutations of the dystrophin gene that lead to the abnormal expression of dystrophin.[134] The disorder of DMD genes includes point mutations, deletions, or duplications, ultimately resulting in the symptoms of muscular dystrophy. In addition to the generation of truncated but functional dystrophin by the NHEJ pathway, the correction of mutated genes by HDR is emerging as a promising therapeutic modality. Lee *et al.*[119] demonstrated that gold nanoparticle-based vectors, termed CRISPR-Gold, can deliver RNP and donor DNA to correct the gene mutations by the pathway of HDR. The generation of CRISPR-Gold nanoparticles combined gold core with thiol-modified oligonucleotides (DNA-Thiol), followed by hybridization of donor DNA. The RNPs were further loaded, before the cationic polymer poly(*N*-(*N*-(2-aminoethyl)-2-aminoethyl) aspartamide) [PAsp (DET)] was covered on the outmost layer. As a result of the cationic nature and the proton-sponge effect, the presence of PAsp (DET) greatly facilitated cellular uptake and the disruption of endosomal membrane to promote intracellular delivery. In the meantime, RNP and donor DNA are released upon exposure to the glutathione-rich milieu. In the study, CRISPR-Gold nanoparticles were injected into tibialis anterior muscle and gastrocnemius to evaluate the gene editing in *mdx* mice. The cryo-sections of CRISPR-Gold treatment group demonstrated good expression of dystrophin protein. Additionally, the CRISPR-Gold treatment group showed less muscle fibrosis than the control groups, as indicated by the trichrome staining of tibialis anterior muscle cryo-sections (Figure 7A, B).[119]

Fragile X syndrome (FXS) is a genetic disease that is a form of autism spectrum disorder (ASD) and intellectual disability. This disease originates in a single gene mutation of fragile X mental retardation 1

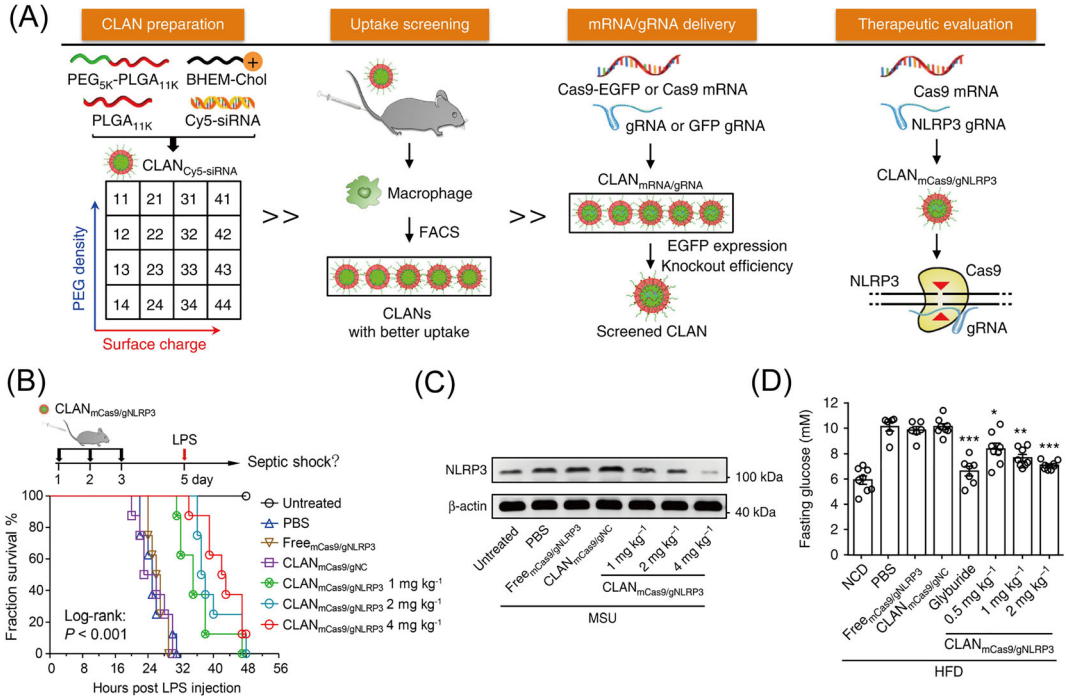

FIGURE 6 Polymer-mediated delivery of Cas9 mRNA for disease therapy. (A) Cationic lipid-assisted polymeric nanoparticle (CLAN)-mediated delivery of Cas9 mRNA (mCas9) and guide RNA (gRNA) into macrophages for inflammatory disease treatment. (B) Mitigation of LPS-induced septic shock via $CLAN_{mCas9/gNLRP3}$-mediated NLRP3 knockout. Therapeutic scheme and survival curve of mice with LPS-induced septic shock. (C) Treatment of MSU-induced peritonitis via $CLAN_{mCas9/gNLRP3}$-mediated NLRP3 knockout. Immunoblot analysis of NLRP3 knockout efficiency in peritoneal macrophages of mice pretreated with $CLAN_{mCas9/gNLRP3}$ or other formulations, and then challenged with MSU. (D) Treatment of HFD-induced T2D via $CLAN_{mCas9/gNLRP3}$-mediated NLRP3 knockout. T2D mice subjected to fasting glucose were treated with $CLAN_{mCas9/gNLRP3}$ or other formulations. Reproduced with permission[121]

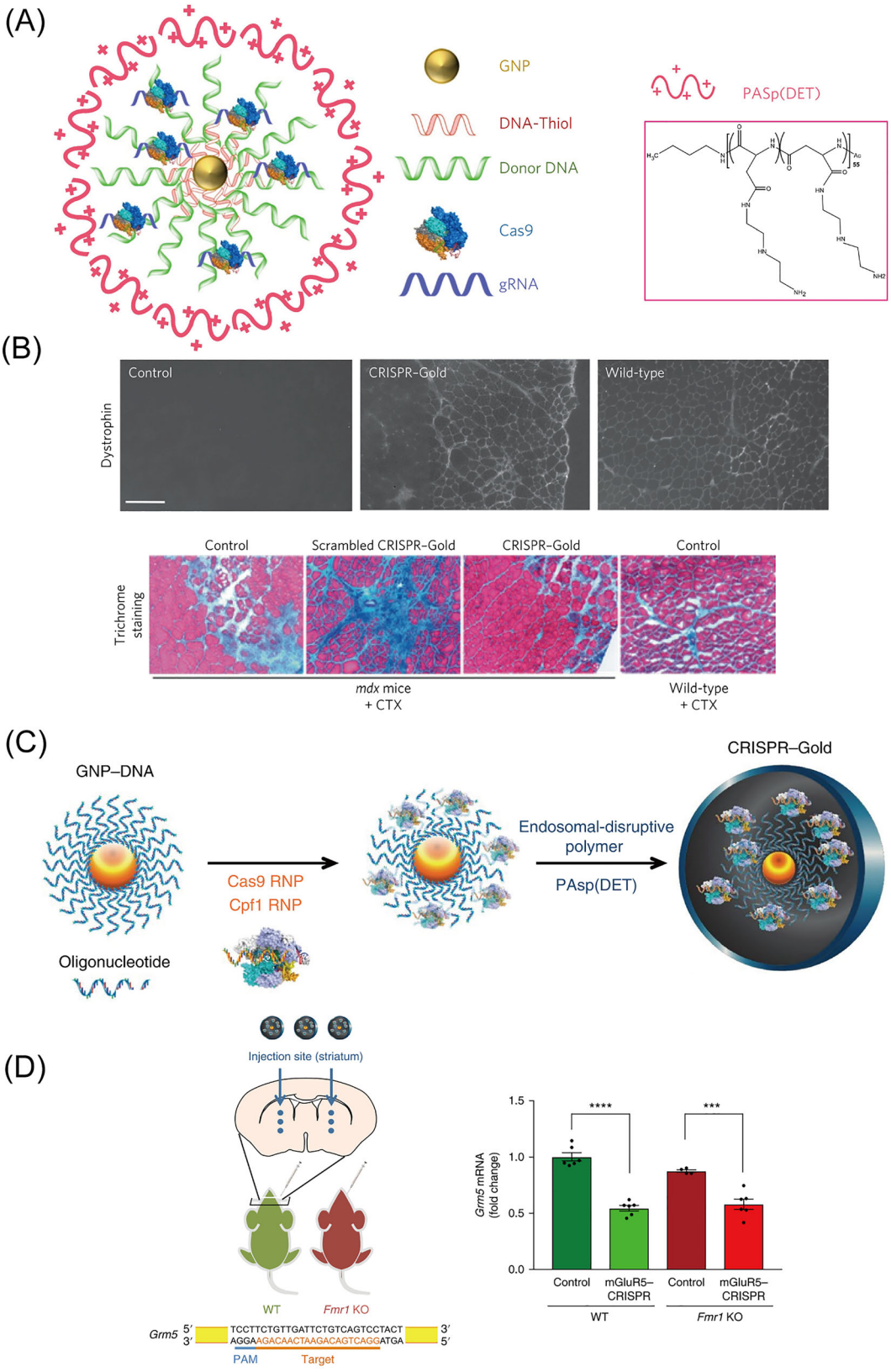

FIGURE 7 Gold nanoparticle-mediated delivery of Cas9 ribonucleoprotein for therapeutic genome editing. (A) Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA *in vivo* induces homology-directed DNA repair. (B) Delivery of CRISPR-gold promotes HDR in the dystrophin gene and dystrophin protein expression, and reduces muscle fibrosis in *mdx* mice, with CTX stimulation. Reproduced with permission.[119] (C) Schematic illustration of CRISPR-gold synthesis. DNA oligonucleotide-conjugated GNPs bind to Cas9 RNPs, and subsequent PAsp (DET) polymer encapsulation generates CRISPR-gold. (D) mGluR5-CRISPR successfully promotes *mGluR5* gene editing in the striatum of wild-type and *Fmr1* knockout mice. Reproduced with permission[120]

(*FMR1*) that leads to the generation of fragile X mental retardation protein. The development of strategies for permanently treating neurological diseases such as FXS is imperative. The *mGluR5* gene served as a target for CRISPR editing with respect to treatment of FXS, because of the exaggerated mGluR5 signaling is closely related to the FXS pathophysiology and other ASDs. To treat FXS, Lee *et al.*[120] used CRISPR-Gold nanocomplexes to deliver Cas9 into the striatum of adult mice brain via local injection. In their study, both wild-type mice and the *Fmr1* knockout mice were treated by injection of saline vehicle or CRISPR-Gold. Quantitative polymerase chain reaction results with reverse transcription revealed the reduction of the mGluR5 mRNA, and the level of mGluR5 mRNA decreased by 40–50% after treatment by CRISPR-Gold. There was also strong evidence that CRISPR-Gold nanoparticles could knock out the *mGluR5* gene in mice. Furthermore, the marble-burying assay and the empty cage observation test were implemented in both wild-type mice and *Fmr1* knockout mice to evaluate the repetitive behaviors. It was found that the symptoms of exaggerated repetitive behaviors were alleviated after the administration of CRISPR-Gold nanoparticles (Figure 7C, D).[120]

6.3 | Liposomes

Lipid nanoparticles play an important role in the delivery of CRISPR/Cas9 elements into cells, and can protect the cargo from degradation to a certain degree.[135] Genome editing by CRISPR/Cas9 could be realized by delivering plasmid DNA, RNA or protein, and all of these formats can be encapsulated into the liposome. sgRNA is negatively charged, whereas Cas9 proteins possess a positive charge. The formation of Cas9 ribonucleoproteins generally generates the negatively charged Cas9/sgRNA complexes as a result of the excessive charge of sgRNA. The electrostatic interaction between lipids and cargo drives the formation of liposome-loaded ribonucleoproteins, to some extent. The cationic liposome has the advantage of an interacting negatively charged cell membrane and this encapsulates the nucleic acids easier. The fusion process, which refers to the interaction between the cationic liposome and cell membrane, is crucial for lipid carriers to release their cargo intracellularly.[136] Commercial transfection reagents, such as Lipofectamine 2000 and Lipofectamine 3000, have been widely applied for the delivery of nucleic acids with excellent transfection capacity. The lipid nanoparticle transfection reagent, Lipofectamine™ CRISPRMAX™ Cas9 (ThermoFisher Scientific), is commercially available for the delivery of CRISPR-Cas9 ribonucleoprotein.

6.3.1 | Liposome-mediated Cas9 plasmid delivery

Cationic lipid nanoparticles decorated with 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (PEG2000-DSPE) were recently designed to encapsulate plasmid encoding Cas9 and sgRNA and obtain nanocomplexes in which the plasmid was pretreated with chondroitin to facilitate its condensation by protamine. The lipid formulation was composed of three components: 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP), dioleoylphosphatidylethanolamine (DOPE), and cholesterol. The nanoparticles were used to deliver Cas9/sgPlk-1 plasmids for editing *Plk1* genes in melanoma tumor-bearing mice and the results obtained demonstrated that it could decrease the expression of Plk1 proteins and restrain the process of tumor growth (Figure 8A, B).[125]

Another delivery platform, which is based on self-assembly between lipid formulation and gold nanoparticles (AuNPs) for the transfection of CRISPR/Cas9 plasmid, was also demonstrated recently. In this study, LACP (lipid/AuNPs/Cas9-sgPlk-1 plasmids) were constructed for therapeutic delivery to knock out *Plk-1* genes in the tumor cells *in vivo*. Gold nanoparticles were first decorated with TAT peptides for both plasmid condensation and nuclear targeting. After loading the plasmid, a lipid layer (consisting of DOTAP, DOPE and cholesterol) was coated on the outer layer and finally decorated with PEG2000-DSPE to form nanoparticles that displayed a lipid-encapsulated shell and a AuNP-condensed Cas9-sgPlk-1 core. The vector showed good ability with respect to delivering plasmids encoding Cas9 and sgRNA into targeted cells. Because the inner gold nanoparticles can harvest near-infrared light and transform this into heat energy, such a delivery system could readily release the payloads by damaging the liposomal layer after light irradiation. Once the cargois released into the cytoplasm, the inner TAT peptides could mediate the delivery of the vector to the target nucleus to facilitate nuclear entry. By irradiating for 20 minutes under a power density of 24 mW cm^{-2}, genome editing could be detected by down-regulating the expression of Plk-1 protein by 65% (Figure 8C, D).[137] Thus, this multifunctional delivery system, and its optically controllable release, provides a useful platform for CRISPR/Cas9 delivery and the treatment of genetic disorders.

6.3.2 | Liposome-mediated Cas9 mRNA delivery

By taking advantage of the merits from viral vectors and non-viral vectors, the combination of both for delivering different CRISPR/Cas9 components would definitely highlight high efficiency and low toxicity, and could avoid the issues of the insufficient packing capacity of viral vectors. Yin *et al.*[138] utilized the combination approach to deliver lipid nanoparticles and AAVs, in which Cas9 mRNA was encapsulated in liposome and sgRNA/HDR template was loaded in AAVs, respectively. After the $Fah^{mut/mut}$ mice were treated with this combinational delivery system targeting *Fah* for 30 days, the experimental groups indicated a significant reduction of liver damage markers, and a stable body weight, compared to the control groups. The results indicated that the initial *Fah* correction in hepatocytes was more than 6%, indicating the great potential of CRISPR/Cas9-based gene therapy to treat hepatic genetic disorders, such as metabolic liver diseases and hemophilia (Figure 9).[138]

6.3.3 | Liposome-mediated Cas9 ribonucleoprotein delivery

To overcome the issues of insufficient gene editing and poor endosomal escape, cationic lipids were also developed to transfer Cas9 proteins and ensure site-specific effective gene editing both

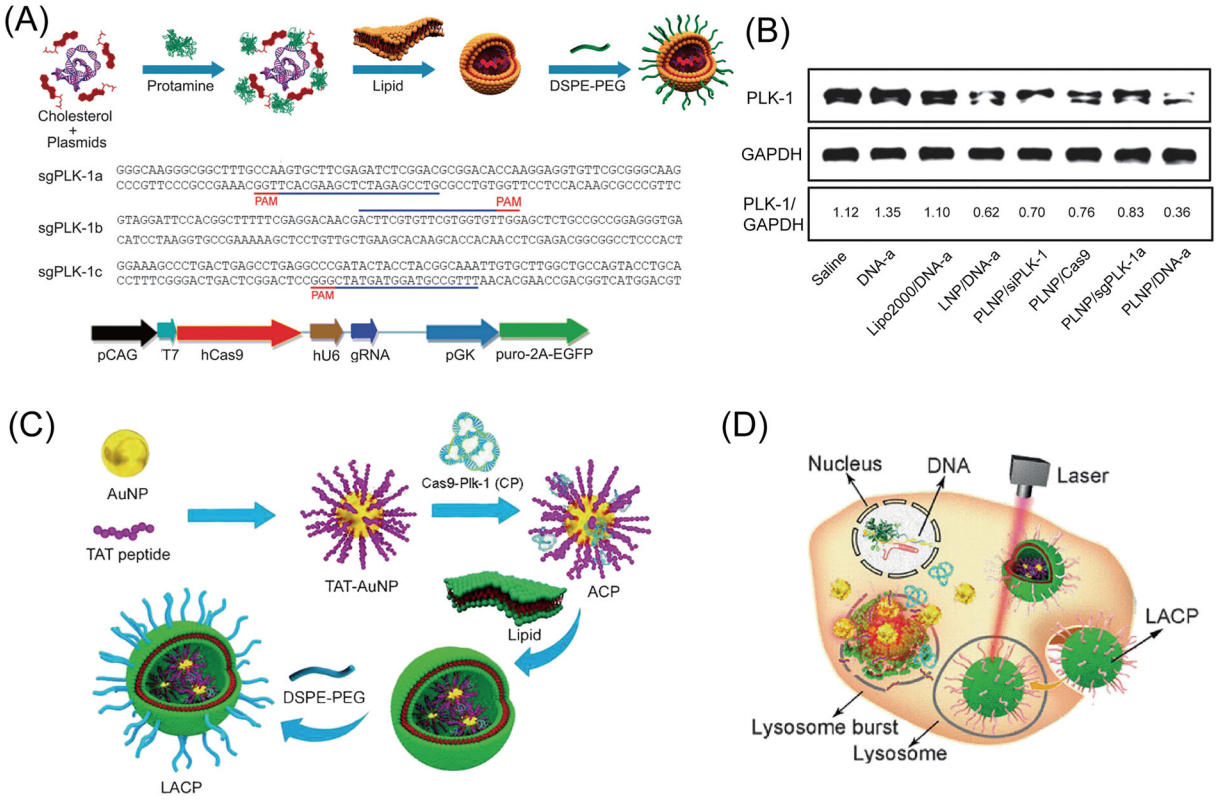

FIGURE 8 (A) Packing and encapsulation processes of the Cas9/sgPlk1-fused plasmid by chondroitin sulfate, protamine, 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP), dioleoylphosphatidylethanolamine (DOPE) and DSPE-PEG. (B) Western blot analysis of *Plk1* protein expression in tumors. Reproduced with permission.[125] (C) Preparation processes for lipid-encapsulated TAT peptide-modified AuNPs (LACPs) and (D) schematic illustration of laser-enhanced knockout of targeted genes by LACP in A375 cells. Reproduced with permission[137]

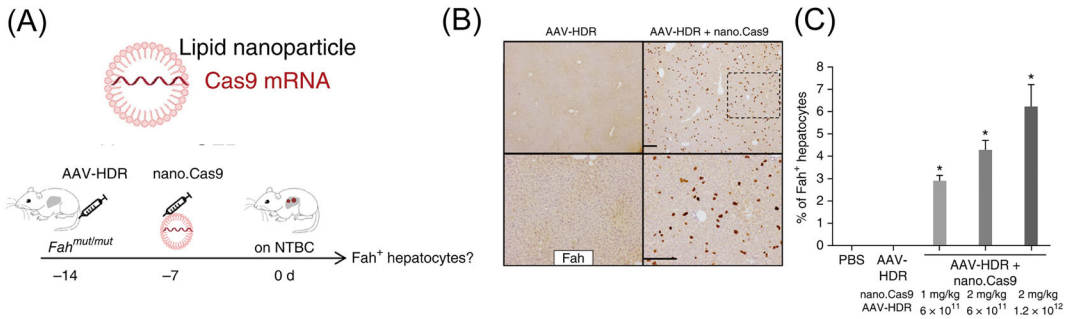

FIGURE 9 Combination of viral and non-viral delivery of CRISPR/Cas9 agents. (A) *In vivo* combined delivery of lipid nanoparticle-encapsulated Cas9 mRNA and AAV-HDR corrects fumarylacetoacetate hydrolase (Fah) mutation. (B) *Fah* immunohistochemistry (IHC). (C) Fah^+ positive cell numbers after the combined delivery. Reproduced with permission[138]

in vitro and *in vivo*. To improve the loading capacity, Cas9 nucleases were first fused with supernegatively charged fusion proteins of (−30)GFP, or complexed with anionic sgRNA. In addition to the guidance function for genome targeting, another critical role of sgRNA is to endow the Cas9/sgRNA complex with sufficient negative charge to facilitate the electrostatic interaction with the liposome. The study also demonstrated that the efficiency of gene editing via liposome-mediated Cas9 nuclease treatment in the inner ear hair cell of mice reached 20% (Figure 10A).[139]

CRISPR/Cas9 system can also be exploited to treat genetic deafness. In a recent study, Gao *et al.*[140] utilized cationic lipids to deliver Cas9 ribonucleoproteins, where sgRNA was designed to target transmembrane channel-like gene family 1 (*Tmc1*) gene in hair cells for ameliorating autosomal dominant hearing loss. Neonatal $Tmc1^{Bth/+}$ mice were treated via injection of nanocomplexes directly into the cochlea. Compared to the control groups, lower thresholds of auditory brainstem response and better levels of acoustic startle response were shown in the experimental groups (Figure 10B).[140]

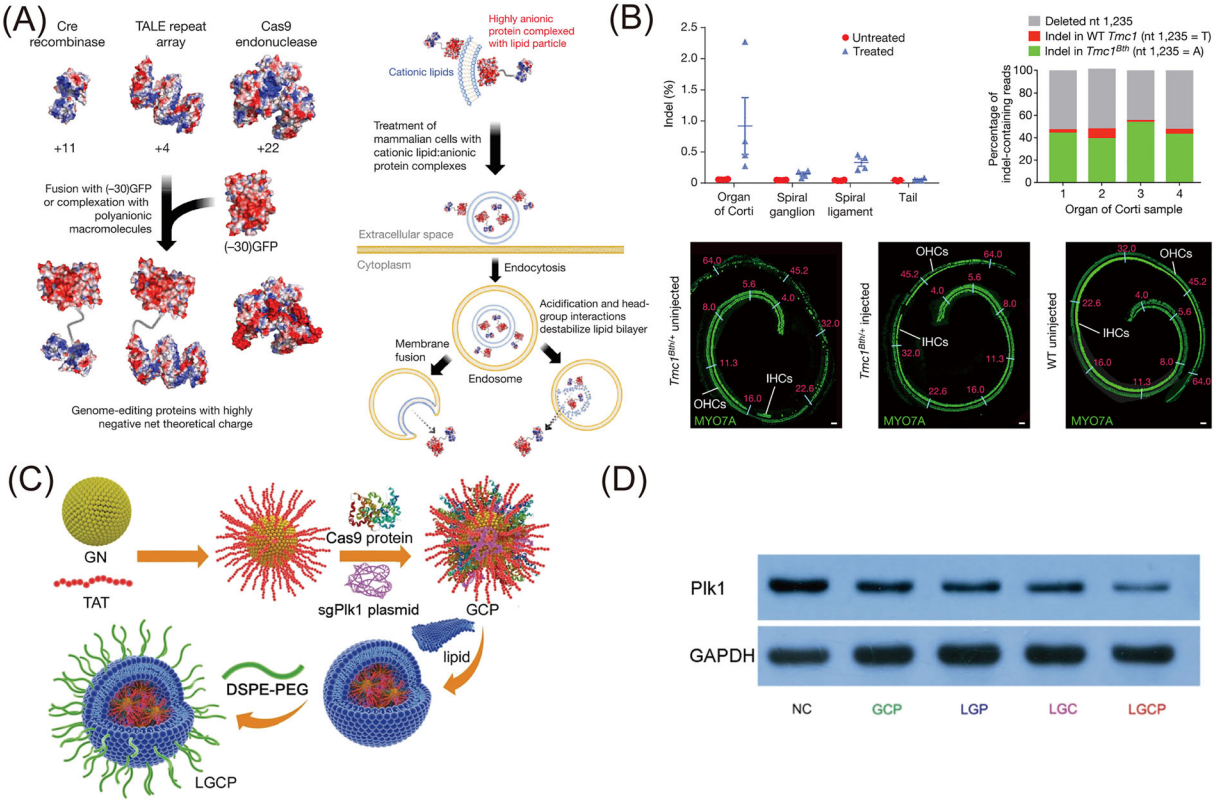

FIGURE 10 (A) Cationic lipid-mediated delivery of Cas9 proteins enables efficient genome editing *in vitro* and *in vivo*. Treatment of autosomal dominant hearing loss.[139] (B) Effects of Cas9 protein–*Tmc1-mut3* sgRNA–lipid injection on hair cell function in mice. Reproduced with permission.[140] (C) Delivery of Cas9 protein/sgRNA plasmid via gold nanocluster/lipid core-shell nanocarriers for melanoma therapy. (D) Western blot analysis of the tumor tissues. Reproduced with permission[124]

Cas9 proteins can also be delivered by lipid-gold nanoparticles for genome editing. Similar to the delivery vector for Cas9 plasmids, gold nanoparticles modified by TAT peptides were constructed to encapsulate both Cas9 proteins and sgRNA plasmids targeting the *Plk1* gene to form nanocomplexes first, followed by the lipid coating (DOTAP, DOPE, and cholesterol), and a fusion process to obtain the lipid-PEG outer layer. A T7 endonuclease I (T7E1) assay suggested that the indel rate reached 26.2%, resulting in an apoptosis rate of 19.4% in A375 cells. The *in vivo* investigation indicated that such a formulation could effectively suppress tumor growth in the melanoma mice. The study provides a powerful, multifunctional, and efficient vector for delivering the CRISPR/Cas9 system for cancer treatment (Figure 10C, D).[124]

6.4 | Cell-penetrating peptides

CPPs comprises of a class of cationic peptides that are highly active with respect to passing through the cell membrane. It is now widely considered that the majority of CPPs and CPP-cargo conjugates enter cells via the endocytosis pathway and they are capable of escaping endosomes by different mechanisms of action.[141] CPPs were initially discovered in viruses and are also characterized as protein transduction domains. As a result of their cell-permeating ability and positive charge, CPPs are usually used to enhance the intracellular delivery efficiency via conjugation with proteins or nucleic acids.[142,143] Typical groups, such as guanidine cations and amine groups, have properties that allow the substrates to be carried into cells by passing through the cell membranes.[144,145] Recently, biodegradable CPPs have been developed to address the release issues. They can release the payloads into the cytoplasm via chemical reaction pathways, such as the reductive hydrolysis of disulfide bonds.[146] Such biodegradable CPPs show a broad prospect for delivering CRISPR/Cas9 components.

Ramakrishna *et al.*[147] reported a carrier-free strategy for delivering Cas9 ribonucleoproteins by conjugating CPPs with Cas9 proteins. To do so, CPPs (termed m9R) were modified with maleimides, and the C-terminal of the Cas9 were modified with cysteine residues. The free thiol residue in the cysteine of Cas9 and the maleimide moiety in CPPs can form thioether bonds by covalent binding (Cas9-m9R). Another positively charged CPP (termed 9R) was complexed with the negatively charged sgRNA (sgRNA-9R), and human cells were sequentially or simultaneously treated with Cas9-m9R and sgRNA-9R. As such, CPP-facilitated delivery of CRISPR/Cas9 did not need any additional carriers or transfection reagents, resulting in efficient genome editing in several human cell lines, including embryonic stem cells, dermal fibroblasts, HEK293T, HeLa, and embryonic carcinoma cells. This carrier-free, CPP-facilitated delivery of CRISPR/Cas9 agents provides a new approach for effective gene editing. In another study, non-covalent conjugation between CPPs and Cas9 was also investigated. In detail, the CPP scaffold consists of arginine, leucines and hydrazide reactive moieties. Because the reactive hydrazide could form the

hydrazine bond with hydrophobic aldehydes, CPPs were thus transformed into amphiphilic penetrating peptides that generated nanoparticles with Cas9 ribonucleoproteins. The interaction between Cas9 ribonucleoproteins and CPPs largely rely on the electrostatic force to drive the formation of Cas9-CPP nanoparticles. Unlike the covalent conjugation, this approach protects Cas9 proteins from being denatured by avoiding harsh chemical reactions and biological engineering processes. The delivery of CPP/Cas9 complexes achieved high gene-editing efficiency in a few types of cell types, including HeLa, A549, and DF1 cells.[148] With low toxicity, this delivery approach appears to be promising for the delivery of Cas9 proteins.

7 | CONCLUSIONS AND FUTURE PERSPECTIVES

With the rapid development of gene-editing technologies, the CRISPR/Cas9 system is becoming a powerful tool for modifying specific genes in eukaryotic cells in a low-cost and user-friendly way. In recent years, CRISPR/Cas9 technology has been widely applied to medicine, biology, genetics, and agriculture, etc. More particularly, it has shown tremendous therapeutic potential for treating single gene disorders, polygenic diseases, and infectious diseases.[118] Clinical trials of CRISPR/Cas9 are also under way. CTX001, an *ex vivo* CRISPR gene-edited therapy, is currently being investigated in clinical phase 1/2 trials for patients suffering from β-thalassemia or sickle cell disease. By increasing the fetal hemoglobin level in the red blood cells from patients, CTX001 is considered to ameliorate symptoms in patients. Moreover, the programmed death-1 (*PD-1*) gene in T cells was knocked out for the treatment of patients with metastatic non-small-cell lung cancer. These studies indicate that CRISPR/Cas9 technologies offer huge therapeutic potential for genetic disorders.[149]

Despite the prospects of CRISPR/Cas9 technologies, several issues still hamper their clinical translation, such as off-target effects, low editing efficiency, and immunogenicity. Particularly, off-target effects have become a major safety concern for CRISPR/Cas9-based therapeutic genome editing. To date, great effort has been made with respect to decreasing off-target effects, and a few possible solutions for reducing such effects are summarized below. The first involves defining the sequence and numbers of sgRNA appropriately to maximize the on-target effects. The second solution is to limit the duration of Cas9 expression so as to reduce the chance of accumulating off-target mutations. The forms of CRISPR/Cas9 delivery also have an important impact on off-target effects. In this respect, Cas9 and sgRNA delivered as a ribonucleoprotein have demonstrated shorter half-times compared to CRISPR/Cas9 plasmids, thereby inducing a lower frequency of mutations at off-target loci.[74,150] As well as reducing off-target effects, it is also important to improve tissue-specific genome editing to reduce side effects, as well as undesirable genome editing. One possible solution is to develop a conditional genome-editing system that only expresses Cas9 with an inducible promoter or a promoter that is only expressed in certain organs. This approach may facilitate the CRISPR/Cas9 plasmids being expressed in certain biological contexts.

The efficiency of gene editing depends on many factors, including the cell type and targeted genome locus. The primary cell is usually hard to transfect, making it difficult for efficient editing.[70,151] Another factor that affects editing efficiency is the delivery strategy. Viral vectors can deliver cargo with a high transfection efficiency, although they suffer from side-effects such as insertional mutagenesis, immunogenicity, and off-target effects. Furthermore, the packaging capacity of viral vectors is usually insufficient to load the CRISPR/Cas9 elements. In this respect, rationally designed non-viral vectors are flexible with respect to packing CRISPR/Cas9 cargo efficiently and are safer to use for therapeutic purposes. Future efforts that aim to improve and optimize the performance of non-viral vectors, and thus attain clinical requirements, are still essential.

In summary, CRISPR/Cas9 gene-editing technology provides the capacity to modify target genes in mammalian cells in a precise and efficient way. The emerging non-viral vectors have brought new opportunities for the delivery of the CRISPR/Cas9 systems, and this at least avoids the side effects and delivery barriers that are confronted by other delivery approaches. Therapeutic genome editing mediated by non-viral delivery vectors represent a promising way of treating numerous genetic disorders. We consider that future advances in non-viral vectors will broaden CRISPR/Cas9-based therapeutic genome editing and provide a promising approach to meet multiple clinical needs.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (Grant No. 81872807), Fundamental Research Funds for the Central Universities (2018XZZX001-14), Thousand Talents Plan (Y. P.), and National Science and Technology Major Project of the Ministry of Science and Technology of China (2018ZX10301402). We also acknowledge Ms. Serena Pun from University of Nottingham for her assistance in the language editing.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

ORCID

Xiaojie Xu https://orcid.org/0000-0002-6607-3339
Yuan Ping https://orcid.org/0000-0003-2571-7721

REFERENCES

1. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iapgene, responsible for alkaline phosphatase isozyme conversion in Escherichiacoli, and identification of the gene product. *J Bacteriol*. 1987;169:5429-5433.

2. Haft DH, Selengut J, Mongodin EF, Nelson KE. A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. *PLoS Comput Biol*. 2005;1:e60.

3. Bolotin A, Quinquis B, Sorokin A, Ehrlich SD. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology*. 2005;151:2551-2561.

4. Pourcel C, Salvignol G, Vergnaud G. CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology*. 2005;151:653-663.
5. Jansen R, Embden JDA, Gaastra W, Schouls LM. Identification of genes that are associated with DNA repeats in prokaryotes. *Mol Microbiol*. 2002;43:1565-1575.
6. Barrangou R, Fremaux C, Deveau H, et al. CRISPR provides acquired resistance against viruses in prokaryotes. *Science*. 2007;315:1709-1712.
7. Horvath P, Barrangou R. CRISPR/Cas, the immune system of bacteria and archaea. *Science*. 2010;327:167-170.
8. Fineran PC, Charpentier E. Memory of viral infections by CRISPR-Cas adaptive immune systems: acquisition of new information. *Virology*. 2012;434:202-209.
9. Wiedenheft B, Sternberg SH, Doudna JA. RNA-guided genetic silencing systems in bacteria and archaea. *Nature*. 2012;482:331-338.
10. Barrangou R, Marraffini LA. CRISPR-Cas systems: prokaryotes upgrade to adaptive immunity. *Mol Cell*. 2014;54:234-244.
11. Van Der Oost J, Westra ER, Jackson RN, Wiedenheft B. Unravelling the structural and mechanistic basis of CRISPR-Cas systems. *Nat Rev Microbiol*. 2014;12:479-492.
12. Bondy-Denomy J, Davidson AR. To acquire or resist: the complex biological effects of CRISPR-Cas systems. *Trends Microbiol*. 2014;22:218-225.
13. Makarova KS, Aravind L, Wolf YI, Koonin EV. Unification of Cas protein families and a simple scenario for the origin and evolution of CRISPR-Cas systems. *Biol Direct*. 2011;6:38.
14. Mohanraju P, Makarova KS, Zetsche B, Zhang F, Koonin EV, Van Der Oost J. Diverse evolutionary roots and mechanistic variations of the CRISPR-Cas systems. *Science*. 2016;353:aad5147.
15. Makarova KS, Wolf YI, Alkhnbashi OS, et al. An updated evolutionary classification of CRISPR-Cas systems. *Nat Rev Microbiol*. 2015;13:722-736.
16. Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science*. 2013;339:819-823.
17. Urnov FD, Miller JC, Lee YL, et al. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature*. 2005;435:646-651.
18. Gabriel R, Lombardo A, Arens A, et al. An unbiased genome-wide analysis of zinc-finger nuclease specificity. *Nat Biotechnol*. 2011;29:816-823.
19. Christian M, Cermak T, Doyle EL, et al. Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics*. 2010;186:757-761.
20. Wu Y, Zhou H, Fan X, et al. Correction of a genetic disease by CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. *Cell Res*. 2015;25:67-79.
21. Cho SW, Kim S, Kim JM, Kim JS. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat Biotechnol*. 2013;31:230-232.
22. Bikard D, Euler CW, Jiang W, et al. Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. *Nat Biotechnol*. 2014;32:1146-1150.
23. Hwang WY, Fu Y, Reyon D, et al. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol*. 2013;31:227-229.
24. Nekrasov V, Staskawicz B, Weigel D, Jones JDG, Kamoun S. Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. *Nat Biotechnol*. 2013;31:691-693.
25. Gasiunas G, Barrangou R, Horvath P, Siksnys V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc Natl Acad Sci USA*. 2012;109:E2579-E2586.
26. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. 2012;337:816-821.
27. Sapranauskas R, Gasiunas G, Fremaux C, Barrangou R, Horvath P, Siksnys V. The *Streptococcus thermophilus* CRISPR/Cas system provides immunity in *Escherichia coli*. *Nucleic Acids Res*. 2011;39:9275-9282.
28. Deltcheva E, Chylinski K, Sharma CM, et al. CRISPR RNA maturation by *trans*-encoded small RNA and host factor RNase III. *Nature*. 2011;471:602-607.
29. Ran FA, Cong L, Yan WX, et al. *In vivo* genome editing using *Staphylococcus aureus* Cas9. *Nature*. 2015;520:186-191.
30. Nishimasu H, Cong L, Yan WX, et al. Crystal structure of *Staphylococcus aureus* Cas9. *Cell*. 2015;162:1113-1126.
31. Garneau JE, Dupuis ME, Villion M, et al. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature*. 2010;468:67-71.
32. San Filippo J, Sung P, Klein H. Mechanism of eukaryotic homologous recombination. *Annu Rev Biochem*. 2008;77:229-257.
33. Rouet P, Smih F, Jasin M. Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Mol Cell Biol*. 1994;14:8096-8106.
34. Bibikova M, Beumer K, Trautman JK, Carroll D. Enhancing gene targeting with designed zinc finger nucleases. *Science*. 2003;300:764.
35. Choulika A, Perrin A, Dujon B, Nicolas JF. Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of Saccharomyces cerevisiae. *Mol Cell Biol*. 1995;15:1968-1973.
36. Bibikova M, Carroll D, Segal DJ, et al. Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. *Mol Cell Biol*. 2001;21:289-297.
37. Krejci L, Altmannova V, Spirek M, Zhao X. Homologous recombination and its regulation. *Nucleic Acids Res*. 2012;40:5795-5818.
38. Plessis A, Perrin A, Haber JE, Dujon B. Site-specific recombination determined by I-SceI, a mitochondrial group I intron-encoded endonuclease expressed in the yeast nucleus. *Genetics*. 1992;130:451-460.
39. Konermann S, Brigham MD, Trevino AE, et al. Optical control of mammalian endogenous transcription and epigenetic states. *Nature*. 2013;500:472-476.
40. Gilbert LA, Larson MH, Morsut L, et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell*. 2013;154:442-451.
41. Shmakov S, Smargon A, Scott D, et al. Diversity and evolution of class 2 CRISPR-Cas systems. *Nat Rev Microbiol*. 2017;15:169-182.
42. Smargon AA, Cox DBT, Pyzocha NK, et al. Cas13b is a type VI-B CRISPR-associated RNA-guided RNase differentially regulated by accessory proteins Csx27 and Csx28. *Mol Cell*. 2017;65:618-630.e7.
43. Konermann S, Lotfy P, Brideau NJ, Oki J, Shokhirev MN, Hsu PD. Transcriptome engineering with RNA-targeting type VI-D CRISPR effectors. *Cell*. 2018;173:665-676.e14.
44. Mout R, Ray M, Lee YW, Scaletti F, Rotello VM. In vivo delivery of CRISPR/Cas9 for therapeutic gene editing: progress and challenges. *Bioconjug Chem*. 2017;28:880-884.

45. Mout R, Ray M, Yesilbag Tonga G, et al. Direct cytosolic delivery of CRISPR/Cas9-ribonucleoprotein for efficient gene editing. *ACS Nano*. 2017;11:2452-2458.
46. Wang T, Wei JJ, Sabatini DM, Lander ES. Genetic screens in human cells using the CRISPR-Cas9 system. *Science*. 2014;343:80-84.
47. Chakraborty S, Ji HY, Kabadi AM, Gersbach CA, Christoforou N, Leong KW. A CRISPR/Cas9-based system for reprogramming cell lineage specification. *Stem Cell Reports*. 2014;3:940-947.
48. Ding Q, Strong A, Pate KM, et al. Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing. *Circ Res*. 2015;115:488-492.
49. Keener AB. Delivering the goods: scientists seek a way to make CRISPR-Cas gene editing more targeted. *Nat Med*. 2015;21:1239-1241.
50. Senís E, Fatouros C, Große S, et al. CRISPR/Cas9-mediated genome engineering: an adeno-associated viral (AAV) vector toolbox. *Biotechnol J*. 2014;9:1402-1412.
51. Yu W, Mookherjee S, Chaitankar V, et al. Nrl knockdown by AAV-delivered CRISPR/Cas9 prevents retinal degeneration in mice. *Nat Commun*. 2017;8:14716.
52. Niu Y, Shen B, Cui Y, et al. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell*. 2014;156:836-843.
53. Xue W, Chen S, Yin H, et al. CRISPR-mediated direct mutation of cancer genes in the mouse liver. *Nature*. 2014;514:380-384.
54. Koike-Yusa H, Li Y, Tan EP, Velasco-Herrera Mdel C, Yusa K. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nat Biotechnol*. 2014;32:267-273.
55. Shalem O, Sanjana NE, Hartenian E, et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science*. 2014;343:84-87.
56. Long C, Amoasii L, Mireault AA, et al. Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science*. 2016;351:400-403.
57. Chen X, Gonçalves MA. Engineered viruses as genome editing devices. *Mol Ther*. 2016;24:447-457.
58. Mátrai J, Chuah MK, Vandendriessche T. Recent advances in lentiviral vector development and applications. *Mol Ther*. 2010;18:477-490.
59. Kotterman MA, Chalberg TW, Schaffer DV. Viral vectors for gene therapy: translational and clinical outlook. *Annu Rev Biomed Eng*. 2015;17:63-89.
60. Qin W, Dion SL, Kutny PM, et al. Efficient CRISPR/Cas9-mediated genome editing in mice by zygote electroporation of nuclease. *Genetics*. 2015;200:423-430.
61. Gori JL, Hsu PD, Maeder ML, Shen S, Welstead GG, Bumcrot D. Delivery and specificity of CRISPR-Cas9 genome editing technologies for human gene therapy. *Hum Gene Ther*. 2015;26:443-451.
62. Tebas P, Stein D, Tang WW, et al. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *N Engl J Med*. 2014;370:901-910.
63. Mali P, Yang L, Esvelt KM, et al. RNA-guided human genome engineering via Cas9. *Science*. 2013;339:823-826.
64. Sharei A, Zoldan J, Adamo A, et al. A vector-free microfluidic platform for intracellular delivery. *Proc Natl Acad Sci USA*. 2013;110:2082-2087.
65. Hendel A, Bak RO, Clark JT, et al. Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nat Biotechnol*. 2015;33:985-989.
66. Kim S, Kim D, Cho SW, Kim J, Kim JS. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res*. 2014;24:1012-1019.
67. Li L, Hu S, Chen X. Non-viral delivery systems for CRISPR/Cas9-based genome editing: challenges and opportunities. *Biomaterials*. 2018;171:207-218.
68. Liu C, Zhang L, Liu H, Cheng K. Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications. *J Control Release*. 2017;266:17-26.
69. Wan T, Niu D, Wu C, Xu FJ, Church G, Ping Y. Material solutions for delivery of CRISPR/Cas-based genome editing tools: current status and future outlook. *Mater Today*. 2019;26:40-66.
70. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc*. 2013;8:2281-2308.
71. Glass Z, Lee M, Li Y, Xu Q. Engineering the delivery system for CRISPR-based genome editing. *Trends Biotechnol*. 2018;36:173-185.
72. Shalem O, Sanjana NE, Zhang F. High-throughput functional genomics using CRISPR-Cas9. *Nat Rev Genet*. 2015;16:299-311.
73. Chen S, Sanjana NE, Zhang F, et al. Genome-wide CRISPR screen in a mouse model of tumor growth and metastasis. *Cell*. 2015;160:1246-1260.
74. Liang X, Potter J, Kumar S, et al. Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. *J Biotechnol*. 2015;208:44-53.
75. Fu Y, Foden JA, Khayter C, et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol*. 2013;31:822-826.
76. Han X, Liu Z, Jo MC, et al. CRISPR-Cas9 delivery to hard-to-transfect cells via membrane deformation. *Sci Adv*. 2015;1:e1500454.
77. Zuckermann M, Hovestadt V, Knobbe-thomsen CB, et al. Somatic CRISPR/Cas9-mediated tumour suppressor disruption enables versatile brain tumour modelling. *Nat Commun*. 2015;6:7391.
78. Hemmi H, Takeuchi O, Kawai T, et al. A toll-like receptor recognizes bacterial DNA. *Nature*. 2000;408:740-745.
79. Chang N, Sun C, Gao L, et al. Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. *Cell Res*. 2013;23:465-472.
80. Shen B, Zhang W, Zhang J, et al. Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. *Nat Methods*. 2014;11:399-402.
81. Chew WL, Tabebordbar M, Cheng JKW, et al. A multifunctional AAV-CRISPR-Cas9 and its host response. *Nat Methods*. 2016;13:868-874.
82. Wang D, Mou H, Li S, et al. Adenovirus-mediated somatic genome editing of pten by CRISPR/Cas9 in mouse liver in spite of Cas9-specific immune responses. *Hum Gene Ther*. 2015;26:432-442.
83. Tenzer S, Docter D, Kuharev J, et al. Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. *Nat Nanotechnol*. 2013;8:772-781.
84. Pillai PP, Huda S, Kowalczyk B, Grzybowski BA. Controlled pH stability and adjustable cellular uptake of mixed-charge nanoparticles. *J am Chem Soc*. 2013;135:6392-6395.
85. Rodriguez PL, Harada T, Christian DA, Pantano DA, Tsai RK, Discher DE. Minimal "self" peptides that inhibit phagocytic clearance and enhance delivery of nanoparticles. *Science*. 2013;339:971-975.
86. Anselmo AC, Modery-Pawlowski CL, Menegatti S, et al. Platelet-like nanoparticles: mimicking shape, flexibility, and surface biology of platelets to target vascular injuries. *ACS Nano*. 2014;8:11243-11253.

87. Jiang S, Cao Z. Ultralow-fouling, functionalizable, and hydrolyzable zwitterionic materials and their derivatives for biological applications. *Adv Mater*. 2010;22:920-932.

88. Mitchell MJ, Wayne E, Rana K, Schaffer CB, King MR. TRAIL-coated leukocytes that kill cancer cells in the circulation. *Proc Natl Acad Sci USA*. 2014;111:930-935.

89. Chaudhuri O, Koshy ST, Cunha CBD, et al. Extracellular matrix stiffness and composition jointly regulate the induction of malignant phenotypes in mammary epithelium. *Nat Mater*. 2014;13:970-978.

90. Sun W, Ji W, Hu Q, Wright G, Gu Z. Leveraging physiology for precision drug delivery. *Physiol Rev*. 2017;97:189-225.

91. Zhang S, Gao H, Bao G. Physical principles of nanoparticle cellular endocytosis. *ACS Nano*. 2015;9:8655-8671.

92. Xu S, Olenyuk BZ, Okamoto CT, Hamm-Alvarez SF. Targeting receptor-mediated endocytotic pathways with nanoparticles: rationale and advances. *Adv Drug Deliv Rev*. 2013;65:121-138.

93. Semple SC, Akinc A, Chen J, et al. Rational design of cationic lipids for siRNA delivery. *Nat Biotechnol*. 2010;28:172-176.

94. Sugahara KN, Teesalu T, Karmali PP, et al. Coadministration of a tumor-penetrating peptide enhances the efficacy of cancer drugs. *Science*. 2010;328:1031-1035.

95. Xing H, Wong NY, Xiang Y, Lu Y. DNA aptamer functionalized nanomaterials for intracellular analysis, cancer cell imaging and drug delivery. *Curr Opin Chem Biol*. 2012;16:429-435.

96. Rosi NL, Giljohann DA, Thaxton CS, Lytton-Jean AK, Han MS, Mirkin CA. Oligonucleotide-modified gold nanoparticles for intracellular gene regulation. *Science*. 2006;312:1027-1030.

97. Sahay G, Alakhova DY, Kabanov AV. Endocytosis of nanomedicines. *J Control Release*. 2010;145:182-195.

98. Hsu CYM, Uludağ H. A simple and rapid nonviral approach to efficiently transfect primary tissue-derived cells using polyethylenimine. *Nat Protoc*. 2012;7:935-945.

99. Benjaminsen RV, Mattebjerg MA, Henriksen JR, Moghimi SM, Andresen TL. The possible "proton sponge" effect of polyethylenimine (PEI) does not include change in lysosomal pH. *Mol Ther*. 2013;21:149-157.

100. Dicarlo JE, Norville JE, Mali P, Rios X, Aach J, Church GM. Genome engineering in saccharomyces cerevisiae using CRISPR-Cas systems. *Nucleic Acids Res*. 2013;41:4336-4343.

101. Davis ME. Non-viral gene delivery systems. *Curr Opin Biotechnol*. 2002;13(2):128-131.

102. Zhou Y, Zhu S, Cai C, et al. High-throughput screening of a CRISPR/Cas9 library for functional genomics in human cells. *Nature*. 2014;509:487-491.

103. Konerman S, Brigham MD, Trevino AE, et al. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature*. 2015;517:583-588.

104. Matano M, Date S, Shimokawa M, et al. Modeling colorectal cancer using CRISPR-Cas9-mediated engineering of human intestinal organoids. *Nat Med*. 2015;21:256-262.

105. Lu XJ, Xue HY, Ke ZP, Chen JL, Ji LJ. CRISPR-Cas9: a new and promising player in gene therapy. *J Med Genet*. 2015;52:289-296.

106. Sánchez-Rivera FJ, Jacks T. Applications of the CRISPR-Cas9 system in cancer biology. *Nat Rev Cancer*. 2015;15:387-395.

107. Maddalo D, Manchado E, Concepcion CP, et al. *In vivo* engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. *Nature*. 2014;516:423-427.

108. Qi LS, Larson MH, Gilbert LA, et al. Resource repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell*. 2013;152:1173-1183.

109. Chen B, Gilbert LA, Cimini BA, et al. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell*. 2013;155:1479-1491.

110. Wu X, Mao S, Yang Y, Rushdi MN, Krueger CJ, Chen AK. A CRISPR/molecular beacon hybrid system for live-cell genomic imaging. *Nucleic Acids Res*. 2018;46:e80.

111. Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol*. 2014;32:347-355.

112. Choi PS, Meyerson M. Targeted genomic rearrangements using CRISPR/Cas technology. *Nat Commun*. 2014;5:3728.

113. Wang H, Yang H, Shivalila CS, et al. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*. 2013;153:910-918.

114. Lombardo A, Genovese P, Beausejour CM, et al. Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat Biotechnol*. 2007;25:1298-1306.

115. Cohen J, Pertsemlidis A, Kotowski IK, Graham R, Garcia CK, Hobbs HH. Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. *Nat Genet*. 2005;37:161-165.

116. Musunuru K, Pirruccello JP, Do R, et al. Exome sequencing, ANGPTL3 mutations, and familial combined hypolipidemia. *N Engl J Med*. 2010;363:2220-2227.

117. Cox DBT, Platt RJ, Zhang F. Therapeutic genome editing: prospects and challenges. *Nat Publ Gr*. 2015;21:121-131.

118. Wang H, Li M, Lee CM, et al. CRISPR/Cas9-based genome editing for disease modeling and therapy: challenges and opportunities for nonviral delivery. *Chem Rev*. 2017;117:9874-9906.

119. Lee K, Conboy M, Park HM, et al. Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. *Nat Biomed Eng*. 2017;1:889-901.

120. Lee B, Lee K, Panda S, et al. Nanoparticle delivery of CRISPR into the brain rescues a mouse model of fragile X syndrome from exaggerated repetitive behaviours. *Nat Biomed Eng*. 2018;2:497-507.

121. Xu C, Lu Z, Luo Y, et al. Targeting of NLRP3 inflammasome with gene editing for the amelioration of inflammatory diseases. *Nat Commun*. 2018;9:4092.

122. Luo YL, Xu CF, Li HJ, et al. Macrophage-specific *in vivo* gene editing using cationic lipid-assisted polymeric nanoparticles. *ACS Nano*. 2018;12:994-1005.

123. Li L, Song L, Liu X, et al. Artificial virus delivers CRISPR-Cas9 system for genome editing of cells in mice. *ACS Nano*. 2017;11:95-111.

124. Wang P, Zhang L, Xie Y, et al. Genome editing for cancer therapy: delivery of Cas9 protein/sgRNA plasmid via a gold nanocluster/lipid core-shell nanocarrier. *Adv Sci*. 2017;4:1700175.

125. Zhang L, Wang P, Feng Q, et al. Lipid nanoparticle-mediated efficient delivery of CRISPR/Cas9 for tumor therapy. *NPG Asia Mater*. 2017;9:e441.

126. Lao YH, Li M, Gao MA, et al. HPV oncogene manipulation using nonvirally delivered CRISPR/Cas9 or *Natronobacterium gregoryi* argonaute. *Adv Sci*. 2018;5:1700540.

127. Gu Z, Biswas A, Zhao M, Tang Y. Tailoring nanocarriers for intracellular protein delivery. *Chem Soc Rev*. 2011;40:3638-3655.

128. Zhang Z, Wan T, Chen Y, et al. Cationic polymer-mediated CRISPR/Cas9 plasmid delivery for genome editing. *Macromol Rapid Commun*. 2019;40:1800068.
129. Liu C, Wan T, Wang H, Zhang S, Ping Y, Cheng Y. A boronic acid-rich dendrimer with robust and unprecedented efficiency for cytosolic protein delivery and CRISPR/Cas9 gene editing. *Sci Adv*. 2019;5:eaaw8922.
130. Wang HX, Song Z, Lao YH, et al. Nonviral gene editing via CRISPR/Cas9 delivery by membrane-disruptive and endosomolytic helical polypeptide. *Proc Natl Acad Sci USA*. 2018;115:4903-4908.
131. Liu Y, Cao ZT, Xu CF, Lu ZD, Luo YL, Wang J. Optimization of lipid-assisted nanoparticle for disturbing neutrophils-related inflammation. *Biomaterials*. 2018;172:92-104.
132. Liang C, Li F, Wang L, et al. Tumor cell-targeted delivery of CRISPR/Cas9 by aptamer-functionalized lipopolymer for therapeutic genome editing of VEGFA in osteosarcoma. *Biomaterials*. 2017;147:68-85.
133. He XY, Liu BY, Peng Y, Zhuo RX, Cheng SX. Multifunctional vector for delivery of genome editing plasmid targeting β-catenin to remodulate cancer cell properties. *ACS Appl Mater Interfaces*. 2019;11:226-237.
134. Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM. Complete loning of the duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell*. 1987;50:509-517.
135. Judge AD, Bola G, Lee ACH, Maclachlan I. Design of noninflammatory synthetic siRNA mediating potent gene silencing *in vivo*. *Mol Ther*. 2006;13:494-505.
136. Basha G, Novobrantseva TI, Rosin N, et al. Influence of cationic lipid composition on gene silencing properties of lipid nanoparticle formulations of siRNA in antigen-presenting cells. *Mol Ther*. 2011;19:2186-2200.
137. Wang P, Zhang L, Zheng W, et al. Thermo-triggered release of CRISPR-Cas9 system by lipid- encapsulated gold nanoparticles for tumor therapy. *Angew Chem Int Ed Engl*. 2018;57:1491-1496.
138. Yin H, Song CQ, Dorkin JR, et al. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components *in vivo*. *Nat Biotechnol*. 2016;34:328-333.
139. Zuris JA, Thompson DB, Shu Y, et al. Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing *in vitro* and *in vivo*. *Nat Biotechnol*. 2015;33:73-80.
140. Gao X, Tao Y, Lamas V, et al. Treatment of autosomal dominant hearing loss by *in vivo* delivery of genome editing agents. *Nature*. 2018;553:217-221.
141. Guidotti G, Brambilla L, Rossi D. Cell-penetrating peptides: from basic research to clinics. *Trends Pharmacol Sci*. 2017;38:406-424.
142. Tezgel AÖ, Gonzalez-Perez G, Telfer JC, Osborne BA, Minter LM, Tew GN. Novel protein transduction domain mimics as nonviral delivery vectors for siRNA targeting NOTCH1 in primary human T cells. *Mol Ther*. 2013;21:201-209.
143. Som A, Reuter A, Tew GN. Protein transduction domain mimics: the role of aromatic functionality. *Angew Chem Int Ed Engl*. 2012;124:1004-1007.
144. Song A, Walker SG, Parker KA, Sampson NS. Antibacterial studies of cationic polymers with alternating, random, and uniform backbones. *ACS Chem Biol*. 2011;6:590-599.
145. Tezgel AÖ, Telfer JC, Tew GN. De novo designed protein transduction domain mimics from simple synthetic polymers. *Biomacromolecules*. 2011;12:3078-3083.
146. Yoo J, Lee DY, Gujrati V, et al. Bioreducible branched poly (modified nona-arginine) cell-penetrating peptide as a novel gene delivery platform. *J Control Release*. 2017;246:142-154.
147. Ramakrishna S, Kwaku Dad AB, Beloor J, Gopalappa R, Lee SK, Kim H. Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. *Genome Res*. 2014;24:1020-1027.
148. Lostalã-Seijo I, Louzao I, Juanes M, Montenegro J. Peptide/Cas9 nanostructures for ribonucleoprotein cell membrane transport and gene edition. *Chem Sci*. 2017;8:7923-7931.
149. Cyranoski D. CRISPR gene-editing tested in a person for the first time. *Nature*. 2016;539:479.
150. Koo T, Lee J, Kim JS. Measuring and reducing off-target activities of programmable nucleases including CRISPR-Cas9. *Mol Cells*. 2015;38:475-481.
151. Hou Z, Zhang Y, Propson NE, et al. Efficient genome engineering in human pluripotent stem cells using Cas9 from Neisseria meningitidis. *Proc Natl Acad Sci USA*. 2013;110:15644-15649.

How to cite this article: Xu X, Wan T, Xin H, et al. Delivery of CRISPR/Cas9 for therapeutic genome editing. *J Gene Med*. 2019;21:e3107. https://doi.org/10.1002/jgm.3107