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Review article

Delivery of CRISPR-Cas tools for *in vivo* genome editing therapy: Trends and challenges

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ABSTRACT

The discovery of clustered regularly interspaced short palindromic repeats (CRISPR) genome editing technology opened the door to provide a versatile approach for treating multiple diseases. Promising results have been shown in numerous pre-clinical studies and clinical trials. However, a safe and effective method to deliver genome-editing components is still a key challenge for *in vivo* genome editing therapy. Adeno-associated virus (AAV) is one of the most commonly used vector systems to date, but immunogenicity against capsid, liver toxicity at high dose, and potential genotoxicity caused by off-target mutagenesis and genomic integration remain unsolved. Recently developed transient delivery systems, such as virus-like particle (VLP) and lipid nanoparticle (LNP), may solve some of the issues. This review summarizes existing *in vivo* delivery systems and possible solutions to overcome their limitations. Also, we highlight the ongoing clinical trials for *in vivo* genome editing therapy and recently developed genome editing tools for their potential applications.

1. Introduction

Genome editing technology utilizes a sequence-specific DNA binding module to introduce a locus-specific DNA insertion, deletion, or alteration in the genome of a living cell. Starting from meganucleases, zinc-finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs), genome editing tools have evolved and become one of the essential tools for gene engineering. Significantly, the finding of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 (CRISPR associated protein 9) as a programmable nuclease in 2012 by Drs. Jennifer Doudna and Emmanuelle Charpentier expanded the possibilities of genome editing even further [1].

The simplicity and accuracy of CRISPR-Cas9 made genome editing much more approachable for correcting genetic mutation or disrupting a target gene. The idea of restoring a genetic mutation has been around for more than 30 years, but classical "gene therapy" can only add or supply a target gene into cells, while genome editing can directly correct or alter a specific locus of the host genome. Here, we use the term "genome editing therapy" as the introduction of genome editing tool(s) into cells to modify endogenous genomic sequence(s) for treating a disease. Genome editing therapy can be a part of gene therapy, as genome editing tools can be introduced as genetic material, but genome editing therapy can significantly extend the applicability in which conventional gene therapy cannot achieve. Both techniques are similar in the way they intervene in a gene or the genomic sequence. Hence, various technologies developed for gene therapy can be applicable for clinical translation of genome editing, including gene delivery technologies for targeting *in vivo* tissues.

The delivery of genome editing tools is one of the major bottlenecks for clinical translation in vivo genome editing [2]. Various methods including, viral and non-viral vectors, have been evaluated in multiple clinical trials. Each vector system has unique strengths and weaknesses that require further improvement to suit the vector for therapeutic applications. Here we summarize the pros and cons of different methodologies for delivering genome editing tools *in vivo*. Some delivery technologies have already been investigated in clinical trials. Lastly, we introduce remaining challenges for genome editing delivery *in vivo* and how the latest genome editing tools may help expand the number of treatable diseases when combined with appropriate delivery tools.

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2. Classification of delivery technologies

2.1. Three forms of CRISPR-Cas cargo

To summarize the various delivery technologies, cargo molecules and carrier types can be classified separately. For example, as a cargo molecule, CRISPR-Cas9 and guide RNA (gRNA) genome editing machinery are delivered as one of the three forms; DNA, RNA, or ribonucleoprotein (RNP, as Cas9 protein works when complexed with gRNA) [3].

The DNA form of delivery requires the DNA to enter the host nucleus for being transcribed into mRNA and then translated into Cas9 protein. Hence, DNA has the slowest expression kinetics than the other two forms [4]. In addition, to express the gRNA from DNA, polymerase III promoters are typically utilized to drive transcription. DNA is relatively stable, and depending on the delivery method, DNA may integrate into the host chromosome or be maintained as an episomal DNA within the nucleus. Thus, if sustained Cas9 expression is necessary, delivery as a DNA form is advantageous to permit long-term expression [3]. However, prolonged expression of Cas9 increases the probability of off-target effects posing safety concerns [5]. Furthermore, the molecular weight of a 10 kb plasmid DNA exceeds 6400 kDa. Such a large molecular size would impede the *in vivo* delivery efficiency.

In the RNA form, Cas9 mRNA (1400 kDa for 4.3 kb mRNA) needs to enter the cytoplasm to be translated into Cas9 protein, along together with gRNA (34 kDa for 0.1 kb RNA). mRNA delivery has faster kinetics than DNA plasmid delivery because it does not need to go through transcription. However, mRNA/gRNA is less stable than DNA and prone to degradation *in vivo* due to endogenous RNases [6]. Thus, chemical modification of RNA has been attempted to improve stability and maximize genome editing efficiency [7].

For RNP, Cas9 protein (160 kDa) is usually combined with gRNA (34 kDa) to form an RNP complex before introducing it into a cell [8]. Since the RNP complex is ready for action, the RNP complex is delivered to the nucleus *via* nuclear localization signal (NLS) and performs genome editing once it enters the cells. Therefore, RNP transduction has the fastest kinetic form of genome editing [6].

2.2. Three major carrier types

Regarding the carrier (or vector) type, it can be categorized into three major groups based on the cellular entry mechanism: (i) biological methods, (ii) chemical methods, and (iii) physical methods (Fig. 1). The biological methods utilize natural biological materials, such as viral protein, peptide, or cellular receptor/membrane to mediate cellular entry. This category includes viral vectors, virus-like particles (VLPs), and cell-penetrating peptides (CPPs). The chemical methods use artificially synthesized materials, such as polymers, lipids, or metal, to catalyze cellular entry. These include liposomes, gold-nanoparticles (AuNPs), and lipid nanoparticles (LNPs). Physical methods rely on the physical energy of electricity or ultrasound to deliver the genes into cells. This category includes electroporation, sonoporation, and microinjection. In the below section, we discuss the different delivery tools based on the carrier type as well as the pros and cons of each technology (Table 1).

3. Biological delivery methods (viral vectors, VLPs, and CPPs)

3.1. Viral vectors

So far, viral vectors seem to be the most efficient delivery method for CRISPR-Cas9 to host cells because viruses have evolved in nature to effectively infect many kinds of tissues and cell types in humans. Also, the virion structure protects the cargo from degradation by host enzymes while in the body or tissue [9]. Once viruses enter target cells, they replicate within the host cells to permit long-term expression.

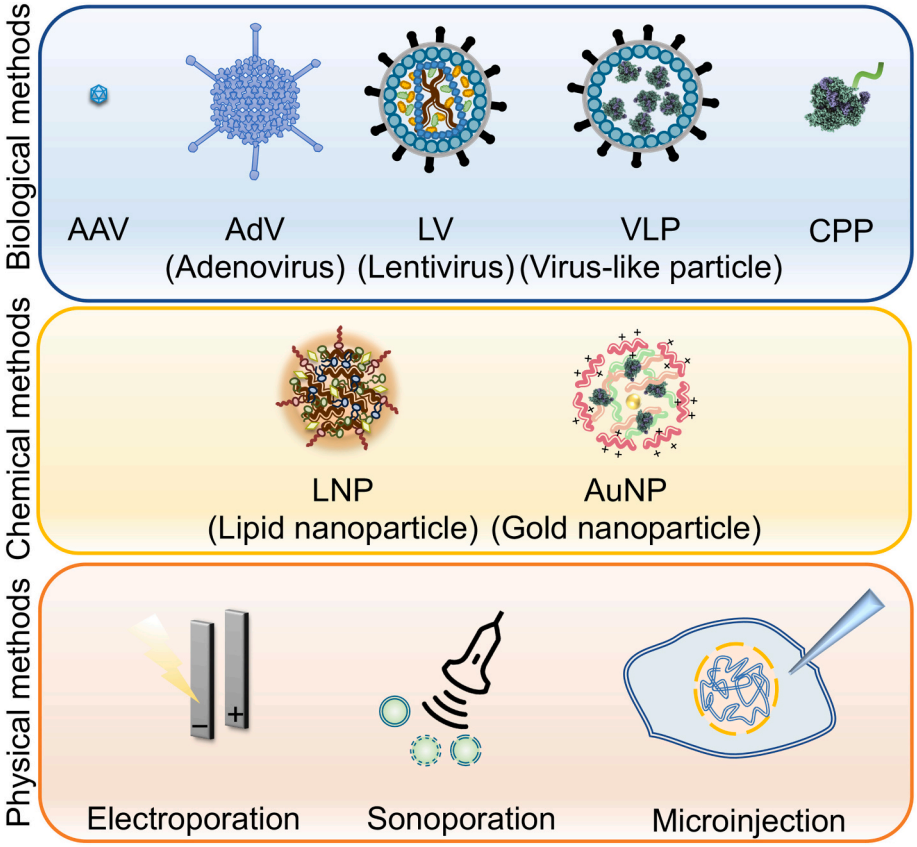

Fig. 1. Various methods for delivering CRISPR-Cas9 components.
The schematic illustrations represent varieties of the tools to deliver genome editing components. The delivery tools are roughly classified into three categories: biological, chemical, and physical methods, based on the constituents and cellular entry mechanisms. For delivering cargo molecules into a cell, biological methods utilize cellular or viral components, chemical methods use chemically synthesized molecules, and physical methods utilize physical energy. AAV; Adeno-associated virus, AdV; adenovirus, LV; lentivirus, VLP; virus-like particles, LNP; lipid nanoparticles, AuNP; gold nanoparticle, CPP; cell-penetrating peptide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Classification of delivery technologies based on delivery method and cargo format.

Cargo format	DNA expression cassette	mRNA for Cas9 translation	Protein form of Cas9
Biological delivery methods	AAV, AdV, LV, IDLV	SeV	VLP, CPP
Chemical delivery methods	Liposome	LNP	AuNP, LNP
Physical delivery methods	Electroporation, Microinjection	Electroporation, Microinjection	Electroporation, Microinjection

AAV; Adeno-associated virus, AdV; adenovirus, LV; lentivirus, IDLV; integrase-deficient lentivirus, SeV; Sendai virus, LNP; lipid nanoparticle, VLP; virus-like particle, CPP; cell-penetrating peptide, AuNP; gold nanoparticle.

Importantly, virulent or disease-causing genes are removed from the vector genome for safety reasons [10]. Depending on the vector type, viral replication genes are also deleted to make the replication of the viral vector incompetent within target cells. Here, we summarize the most widely used viral vectors, such as adeno-associated virus (AAV) vectors, adenovirus (AdV) vectors, and lentivirus (LV) vectors.

3.2. Adeno-associated virus (AAV) vector

AAV is a small (20–25 nm in diameter), non-enveloped virus with a single-stranded DNA genome of 4.7 kilobases (kb) in length [11]. AAV is one of the most widely used vectors for *in vivo* gene therapy. AAV can infect a wide range of dividing and quiescent cells, depending on their serotypes [12]. About 11 serotypes and more than 100 variants have been identified in nature, but each serotype broadly differs in tissue tropisms [13,14]. In addition, the AAV genome can be maintained as an episome in non-dividing cells, resulting in continuous expression of the transgene for several years [15].

AAV-mediated delivery of CRISPR-Cas9 has been used in animal models successfully to treat a wide array of disease models, such as phenylketonuria [16], ornithine transcarbamylase deficiency [17], amyotrophic lateral sclerosis [18], familial hypercholesterolemia [19], hemophilia B [20], and Huntington's disease [21].

For retinal disorders, Nishiguchi et al. used the AAV-mediated delivery of the CRISPR-Cas9 and a donor template to replace a mutated sequence causing retinal dystrophy with its wildtype counterpart [22]. In this study, they rescued approximately 10% of the photoreceptors in blind mice, resulting in improved light sensitivity and robust recovery of the visual function [22]. Furthermore, several studies utilized the AAV vector-mediated CRISPR delivery to disrupt disease-causing genes for the treatment of age-related macular degeneration and other neovascularization-associated diseases [23], retinal degeneration [24], and retinal angiogenesis [25], or to remove intronic mutation and restore the gene function as in Leber congenital amaurosis [26,27].

For skeletal muscle disorders, AAV9 and AAV6-mediated CRISPR editing has been applied to restore muscle function for Duchenne muscular dystrophy (DMD) in mouse models [28–32]. Most recently, Min et al. demonstrated that intramuscular delivery of AAV9 containing the CRISPR-Cas9 components restored dystrophin expression efficiently in DMD mice models by inducing exon skipping and reframing [29].

Recently, Tabebordbar and his colleagues have reported engineered AAV by inserting a random peptide into the AAV capsid [33]. After various screenings in mouse models and optimization, they identified a modified AAV capsid that permits highly efficient targeting of skeletal muscle tissue, named "myotube AAV" or "MyoAAV." The MyoAAV successfully delivered the CRISPR-Cas9 system to mice and non-human primates muscle cells and repaired dysfunctional copies of the dystrophin gene in a mouse model. The team showed that MyoAAV is ten times more efficient than AAV9 at reaching muscle cells [33]. Thus, MyoAAV may be used at lower doses than other AAV serotypes for more effective gene therapies while reducing the risk of liver damage and other critical side effects.

3.3. Clinical application of AAV vector

Building upon the many successes with genome editing in animal models, the first clinical trial of genome editing therapy (NCT03041324) led by Sangamo Therapeutics took place in November 2017 using AAV delivery of ZFN (Table 2). This Phase 1/2 clinical trial attempts to treat MPS II (mucopolysaccharidosis II) patients with mutations in the iduronate-2-sulfatase (*IDS*) gene by inserting a correct copy of the *IDS* gene into the albumin locus in hepatocytes. This single-shot infusion aims to provide targeted insertion *via* homology-directed repair, making AAV a suitable supplier of a repair template because its genomic DNA retains as episome within cells. In addition, the ease of AAV reaching the liver makes it a favorable vector.

Similar clinical trials were performed for MPS I (NCT02702115), which is caused by mutations in the *IDUA* gene, and for hemophilia B (NCT02695160) patients, which is caused by mutations in the factor IX (FIX) gene. Unfortunately, all of the three clinical trials failed to demonstrate clinical benefit, probably due to low genome editing activities attributed to either suboptimal activity of the first-generation ZFNs, low activity of HDR mediated insertion, or insufficient delivery by the AAV vector. Detailed analysis and scientific publication are awaited.

For retinal disorder, a phase 1/2 clinical trial (NCT03872479) was performed by Editas Medicine (Table 2). Homozygous or compound heterozygous mutation of c.2991 + 1655A > G in intron 26 (IVS26) of the *CEP290* gene, which generates the abnormal splice donor site, is one of the major causes for Leber congenital amaurosis type 10 (LCA10). CRISPR-Cas9/gRNA was designed to disrupt the abnormal splice donor site to correct the protein reading frame of the *CEP290* gene and delivered to the retina through an AAV vector ($0.6–1.1 \times 10^{12}$ v.g./kg). Few patients responded to the treatment, but the details of the results are being corrected and not published yet [34,35]. Since the eye is a relatively small organ and is known to be an immune privilege site, targeting the eye with an AAV vector is reasonable to treat by one shot and to avoid acute immune responses.

3.4. Limitations of AAV vector

Next, we address the major limitations of the AAV vectors in genome editing therapy. These limitations include immunogenicity, liver toxicity, prolonged Cas9/gRNA expression, and integration of viral genomic sequence.

Immunity against the AAV capsid is a major limitation of the AAV vector, partly because AAV is a non-enveloped virus covered with a protein shell. Therefore, it is easy for the host immune response to create neutralizing antibodies. Such anti-capsid antibodies neutralize AAV particles even at relatively low titers (1:5–1:7), thereby blocking the entry into target cells [44,45]. This means AAV vector-mediated delivery is basically a one-shot therapy and cannot be repeatedly administrated unless a different serotype is used. In addition, pre-existing neutralizing antibodies against AAVs have been detected in many human populations (35– 80%) [46,47]. This limits the utility of the AAV vector, as the AAV vector cannot be used for patients with neutralizing antibodies.

Immunogenicity against AAV can be solved by only enrolling patients negative for neutralizing antibodies, but this approach is not ideal since this excludes a large population [48]. Another suggested approach is by engineering the AAV capsid (*i.e.*, chimeric AAV capsids or altering the antigen site), which was found to escape the humoral immunity partially [49]; however, modifying the AAV capsid may negatively impact the infectivity or tissue tropism [50]. Considering the cross-

Table 2
Ongoing clinical trials for *in vivo* genome editing therapy.

Description	Clinical Trials ID, Phase, Start Year	Delivery Method	Affiliation	Ref.
Inserting a normal copy of *IDUA* gene in hepatocytes to target MPS I patients using zinc-finger nuclease (SB-318)	NCT02702115 Phase 1 / 2 2016	AAV2/6 *via* intravenous (IV) infusion	Sangamo Therapeutics, USA	[36]
Inserting a normal copy of *F9* gene in hepatocytes to target hemophilia-B using zinc-finger nuclease (SB-FIX)	NCT02695160 Phase 1 2016	AAV2/6 *via* intravenous (IV) infusion	Sangamo Therapeutics, USA	[37]
Disrupting *E7* oncogene from HPV to target cervical cancer using zinc-finger nuclease	NCT02800369 Phase 1 2016	Suppository containing ZFN-603 or ZFN-758 *via* intratumoral injection	Huazhong University of Science and Technology, China	[38]
Disrupting *E6/E7* oncogene from HPV to target cervical cancer using TALEN	NCT03226470 Phase 1 2017	Suppository containing T27 and Suppocire *via* intravaginal injection	Huazhong University of Science and Technology, China	[39]
Disrupting *E6/E7* oncogene from HPV to target cervical cancer using TALEN and CRISPR-Cas9	NCT03057912 Phase 1 2017	A gel containing TALEN or CRISPR-Cas9 plasmid, C32–447, Poloxmer 407 *via* intravaginal injection	Sun Yat-Sen University, China	[40] [39]
Inserting correct *IDS* gene in hepatocytes to target MPS II patients using zinc-finger nuclease (SB-913)	NCT03041324 Phase 1 / 2 2017	AAV2/6 *via* intravenous (IV) infusion	Sangamo Therapeutics, USA	[41]
Correcting *CEP290* gene in retinal to target LCA10-IVS26 patients using CRISPR-SaCas9 (EDIT-101)	NCT03872479 Phase 1 / 2 2019	AAV5 *via* subretinal injection	Editas Medicine, Inc., USA	[27]
Clearing HSV infection targeting herpetic stromal keratitis	NCT04560790 Phase 1 / 2 2020	VLPs *via* corneal injection	Shanghai BDgene, China	[42]
using CRISPR-SpCas9 (BD111)				
Knockout *TTR* gene in hepatocytes to target ATTR amyloidosis patients using CRISPR-spCas9 (NTLA-2001)	NCT04601051 Phase 1 2020	LNPs *via* intravenous administration	Intellia Therapeutics, UK	[43]

reactivity of neutralizing antibodies against the AAV capsid among different serotypes may restrict the success of this strategy [50]. Plasmapheresis by temporarily replacing donor plasma with saline or albumin buffer has been considered to reduce or washout anti-AAV antibodies from the blood plasma of patients [51]. However, it may require several cycles of plasmapheresis to achieve a significant decline in antibody titers [51]. Alternatively, the administration of high AAV doses has been proposed to overwhelm the inhibitory effect by neutralizing antibodies, but even low levels of antibodies could inhibit high amounts of AAV [45]. Also, the increased risk of toxicity accompanied by the large doses must be kept in mind [52].

Important to note, liver toxicity of AAV is one of the most concerning side effects associated with a high dose of administration. It has shown that nonhuman primates developed severe hepatotoxicity and morbidity within 4–5 days after intravenous injection of high dose ($\geq 7.5 \times 10^{13}$ v.g./kg) AAV vector [52]. The observed toxicity was related to the extremely high copy number (> 1000 v.g.) of vector genomes in the liver.

The concern came to reality in a gene therapy clinical trial. Hepatotoxicity has been observed in three patients who passed away within 3 to 4 weeks after intravenous administration of a high dose (3×10^{14} v.g./kg) of AT-132 in an AAV gene therapy for the treatment of X-linked myotubular myopathy (XMTM). XMTM is a genetic neuromuscular disease characterized by extreme muscle weakness, severe hypotonia, respiratory failure, and early death. Some patients also developed progressive cholestatic hepatitis and subsequent decompensated liver failure. Two of the patients who received the AT-132 died of sepsis, whereas the third patient succumbed to a gastrointestinal bleed. Although the participants had a medical history of a pre-existing hepatobiliary disease, they demonstrated normal liver ultrasound and bilirubin levels before the AAV administration [53]. Furthermore, the fourth patient who enrolled in the same clinical trial and even received a lower dose (1.3×10^{14} v.g./kg) of AT-132 also died after the weeks of dosing [54]. The study revealed that the patient had abnormal liver tests, but the exact cause of death is still being investigated [54].

Although the cause of death is still under investigation, it appears that all four patients share similarities of having a history of liver diseases. Importantly, AAV vectors are based on non-pathogenic viruses and have been used in hundreds of gene therapy clinical trials successfully and well-tolerated in most cases. Though every drug has toxicity at overdose, the unfortunate consequence that happened in the AT-132 case teaches us to be more cautious in such factors as dose limit, patient condition, as well as other possibilities including vector quality (*i.e.*, amount of empty particles), and potential toxicity of transgene.

The property of a drug is determined by the carrier and the combination with the cargo. AAV vectors typically do not integrate into the host chromosomes. However, in the case of the combination with

CRISPR-Cas9 transgene, recent research demonstrated the high incidence of random integration of AAV genomic fragment [55,56]. When mice were treated with AAV-Cas9, insertion of vector DNA was detected not only at the on-target of Cas9/gRNA but also throughout the chromosomal locations randomly. The possibility of AAV-Cas9 DNA integration might lead to unwanted genotoxicity, such as disruption of an essential endogenous gene (*i.e.*, tumor suppressor gene) or activation of an oncogene if promoter or enhancer sequence was inserted nearby [56].

Prolonged expression of Cas9 from the AAV vector is another concern for genomic toxicity. According to gene therapy-related clinical trials, transgene expression from an AAV vector is known to persist for up to 10 years in humans [15]. Considering Cas9 is a nuclease, not only prolonged expression is unnecessary, but it can also induce unwanted genomic mutagenic, so-called off-target effect [15]. Therefore, minimizing the duration of Cas9 expression would diminish the risk of off-target effect. For this, self-cleaving systems of AAV vector have been investigated to reduce the expression duration in mouse liver [57].

Apart from safety concerns, there is a packaging limitation of the AAV vector, which would affect the efficacy and choice of the cargo molecule. Due to the small packaging capacity of the AAV, which is 4.7 kb, the most widely used *Streptococcus pyogenes* (SpCas9, 4.1 kb) cannot be packaged together with other necessary components, such as gRNA, promoter sequence, and polyadenylation signals [58]. Therefore, in most cases, SpCas9 and gRNA are packaged into two separate AAV vectors [59]; however, this comes at the cost of efficiency [60]. Alternatively, smaller Cas9 orthologs can be packaged into the AAV vector (Fig. 2). Such smaller Cas9 protein orthologs include: Cas9 from *Staphylococcus aureus* (SaCas9; with 3.2 kb) [61], or from *Campylobacter jejuni* (CjCas9; 2.95 kb) [62]. Moreover, two other Cas-family proteins, Cas12a (also known as Cpf1) from *Lachnospiraceae bacterium* or *Acidaminococcus sp.* [23] and Cas12b from the mutant *Bacillus hisashii* (BhCas12b; 3.3 kb) [63], showed a robust genome editing in human cells comparable to SpCas9. Similarly, the CasX (Cas12e; 2.9 kb) from the groundwater bacteria (Deltaproteobacteria and Planctomycetes) induces programmable genome editing in cultured human cells.

3.5. Adenoviral vector (AdV vector)

Human adenoviruses (AdVs) are non-enveloped viruses with an icosahedral-shaped capsid of 90–100 nm diameter, and it has a double-stranded DNA genome of 26–45 kb [64]. Most AdVs cause mild infection (such as fever or cough) in humans, but some may cause life-threatening multi-organ dysfunctions/diseases for immunocompromised individuals [65,66]. At least 57 serotypes of human AdV have been recognized, but most of the AdV vectors are commonly derived from serotypes 2 and 5, which are less pathogenic [64]. AdV vectors have emerged as highly promising vectors for gene delivery development because: (1) of their high transduction efficiency due to broad tissue tropism, (2) ease of high titer production, (3) low pathogenicity in immunocompetent individuals, (4) large cargo capacity up to 38 kb, and (5) it does not integrate into the host genome and reside as episomal DNA [64]. Transgene expression from AdV is transient and typically lasts for several days, which is another advantage for CRISPR-Cas9 delivery to minimize off-target mutagenic risk.

The AdV can also be used for a targeted knock-in approach. Stephens et al. utilized the AdV-mediated delivery of CRISPR-Cas9 to insert the coagulation factor IX (*F9*) gene at the *ROSA26* safe harbor locus in the hemophilia B mice model [67]. They found that a single injection of AdV vectors mediated corrective knock-in of the mouse *F9* cDNA gene at the *ROSA26* locus, resulting in long-term augmentation of F9 protein activity and phenotypic correction in the hemophilia B mice model.

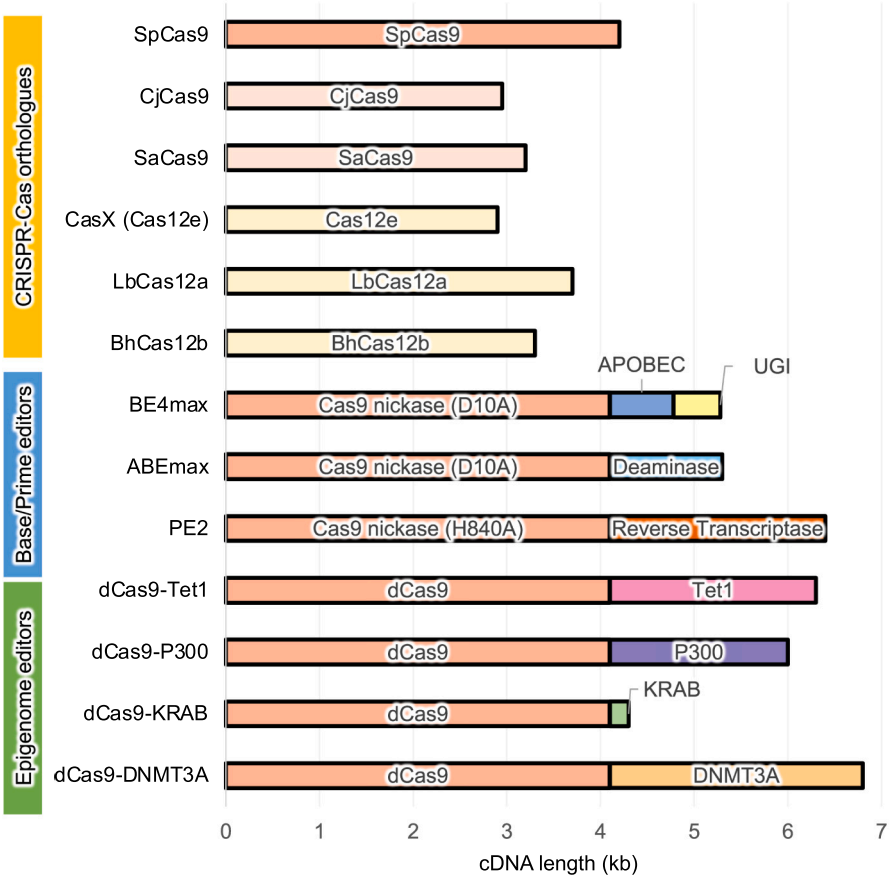

Fig. 2. Size of common CRISPR-Cas9 orthologs and genome editing tools.
The length of each bar represents its corresponding component in cDNA kilobases. The smaller size of Cas9 orthologs are advantageous for delivery purpose; however, the requirement of complex PAM sequence and low or variable activity of DNA cleavage needs to be investigated. Base editors, Prime editors, and epigenome editors are attractive tools for modifying genomic sequence or epigenomic status without introducing a double-strand break (DSB), but they come with a cost of large size; hence the delivery method needs to be optimized.

However, adaptive immune responses against the AdV and Cas9 were detected [67].

Despite such remarkable features that AdV offer, their immunogenicity is a significant concern. This is a favorable feature for vaccine development, but it can induce potential risk (*i.e.*, anaphylaxis) in the case of gene delivery [64]. Due to the high immunogenicity, delivery efficiency can be attenuated from the second injection and onward; hence repeated administrations are ineffective [68]. In addition, ~90% of human populations have pre-existing antibodies. This is why the AdV vaccine for COVID-19 by AstraZeneca is based on chimpanzee AdV. So far, there is no report of the use of the AdV vector in a clinical trial of genome editing.

3.6. Lentiviral vector (LV vector)

Lentiviruses (LVs) are enveloped viruses with 80–120 nm diameter and contain two copies of a single-stranded genomic RNA of 9 kb, which is later reverse-transcribed into double-stranded DNA for integrating into the host chromosome [69]. Multiple LV systems have been developed from human immunodeficiency virus type 1 (HIV-1) [69], simian immunodeficiency virus [69], feline immunodeficiency virus [70], and equine infectious anemia virus [69]. LV vectors have been used mainly for *ex vivo* gene therapy purposes, such as stable integration of CAR (chimeric antigen receptor) cDNA into T cells. This is because LVs have multiple features, including large genetic capacity (up to 8 kb), the broad tropism by VSV-G pseudotyping, and the ability to infect dividing and non-dividing cells [71]. In addition, LVs are less immunogenic than AAV or AdV, as it is covered by a cellular lipid-bilayer, not a viral capsid [72].

Despite multiple advantageous features, the integrating and long-lasting nature of transgene expression makes LV unfavorable for delivering CRISPR-Cas9. The random integration of the viral DNA may lead to either activation of an oncogene [73] or suppression of a tumor suppressor gene [74], which potentially leads to oncogenesis. Furthermore, stably expressing Cas9 and gRNA may lead to a higher risk of off-target mutagenesis [75].

To overcome the genotoxicity issue of LVs, integrase-defective LV (IDLV) vectors have been used for episomal delivery of genome editing machinery [76]. For example, Uchida et al. have used the IDLV vector to deliver CRISPR components into human umbilical cord blood-derived erythroid progenitor-2 (HUDEP-2) cells to correct the sickle cell disease mutation in the β-globin (*HBB*) gene [77]. Although IDLV might be used for *in vivo* applications of CRISPR-Cas9 delivery, the risk of DNA integration at the Cas9 cleavage site should be investigated, considering similar findings observed in AAV-Cas9 studies.

Another concern associated with LV or IDLV is the possibility for the generation of replication-competent lentiviruses (RCLs) due to the recombination with wild-type HIV in the case of HIV-infected individuals. To minimize the likelihood of RCL generation, a minimal number of LV vector-producing genes are separately coded onto multiple plasmids to minimalize homology sequence [78]. Therefore, it is advantageous to remove as many viral genes as possible from the delivery vector to improve safety further.

3.7. Virus-like particle (VLP)

To overcome the challenges associated with the LV vector, we and others have developed LV based virus-like particle (VLP) system that utilizes minimal viral components. VLP is produced through the self-assembly of viral structural proteins [79]. The appearance of VLP is similar to a native virus, but they lack the viral genome, so they are entirely replication-incompetent [80]. As VLP does not contain viral genomic nucleic acid and does not replicate in the host cell, we would like to categorize the VLP system as a form of non-viral delivery technology. VLP had little room for gene argumentation therapy due to its short-lifetime nature. However, VLP has received much attention recently due to its potential for vaccine development [81] and for delivering the CRISPR-Cas9 system transiently for genome editing therapy. Currently, there is one ongoing clinical trial (NCT04560790) using VLP targeting herpetic stromal keratitis conducted by Shanghai BDgene, although specific details are not provided yet (Table 2).

Depending on the delivery vector, Cas9 expression can either be transient or prolonged. This leads to a conceptual difference between the "Hit & Stay" and "Hit & Away" model that should be thought about the kinetics of genome editing (Fig. 3). Most drugs follow the "Hit & Stay" mode, where the drug's half-life correlates with the effective time of the drug. Thus, drug therapeutics are designed to be sustainable so that the effects are long-lasting. However, with genome editing, the "Hit & Away" mode is favored because the short half-life of CRISPR or other editing tools is usually sufficient to produce long-lasting effects. In addition, adverse effects that can be encountered with a prolonged expression of Cas9, such as off-target effects, can be avoided. As a result, transient delivery methods like VLP and other non-viral vehicles are becoming desired for therapeutic application compared to other traditional viral vectors (Table 3).

Several VLP systems have been developed recently, including Cas9P LV that pre-package Cas9 protein in lentiviral particles [82], VEsiCas [83] system that passively incorporates SpCas9, NanoBlades [84] system that fuses SpCas9 with retroviral Gag, or Gesicle system that uses dimerization based incorporation of SpCas9 [85]. As another example of a VLP system, we developed the NanoMEDIC system, which shows the capability of transient genome editing delivery *in vivo*. The NanoMEDIC system utilizes two distinct homing mechanisms to package CRISPR-Cas9 protein and sgRNA, respectively [86]. For Cas9 packaging, chemically inducible heterodimerization domains are attached to Cas9 and HIV Gag. This chemical-based system allows efficient packaging of Cas9 into budding Gag particles in producer cells and allows efferent release of Cas9 from Gag in target cells due to the absence of dimerization inducer. For packaging the gRNA, we utilize an Ψ packaging signal from HIV to direct sgRNA flanked by hammerhead (HH) and hepatitis delta virus (HDV) self-cleaving ribozymes, so that gRNA is packaged into Gag particle *via* the packaging signal and then ribozyme cleavage to free the gRNA for complexing with Cas9. The results revealed that NanoMEDIC could efficiently induce genome editing in various hard-to-transfect human cell types, including T cells, monocytes, iPSCs, and iPSCs-derived neurons. Also, the NanoMEDIC system achieved over 90% exon skipping efficiencies in skeletal muscle cells derived from DMD patient iPSCs. Moreover, a single intramuscular injection of NanoMEDIC induced stable genomic exon skipping in a luciferase reporter mouse and DMD mice model (*mdx*), indicating its utility for *in vivo* genome editing therapy [86].

3.8. Cell-penetrating peptide and nuclear localization signal

Cell-penetrating peptides (CPPs) are another potential tool that enables the delivery of Cas9 RNP directly into cells. CPPs are short peptides, typically less than 30 amino acids long, and can be fused or conjugated with a target molecule to penetrate cellular membranes [87]. In addition, CPP can deliver a wide variety of cargo molecules into living cells, such as proteins, peptides, nanoparticles, siRNAs, liposomes, and drugs [88].

Recently, Gustafsson et al. have utilized an RNA-delivering CPP, PepFect14 (PF14), to deliver Cas9 ribonucleoprotein (RNP). Results showed high editing efficiency of the RNP-CPP complex in HEK293T cells up to 80%. Furthermore, the CPP-RNP complex was highly stable during freeze-thaw cycles and freeze-drying, without loss of editing efficiency [89]. In addition, Kim et al. showed that the fusion of Cas9 with 30Kc19 peptide (containing a CPP of 13 amino acids from a silkworm) efficiently delivered Cas9 into human cells and led to an efficient genome editing [90]. Moreover, the 30Kc19-fused Cas9 protein displayed higher stability against thermal and chemical-induced inactivation than native Cas9 [90].

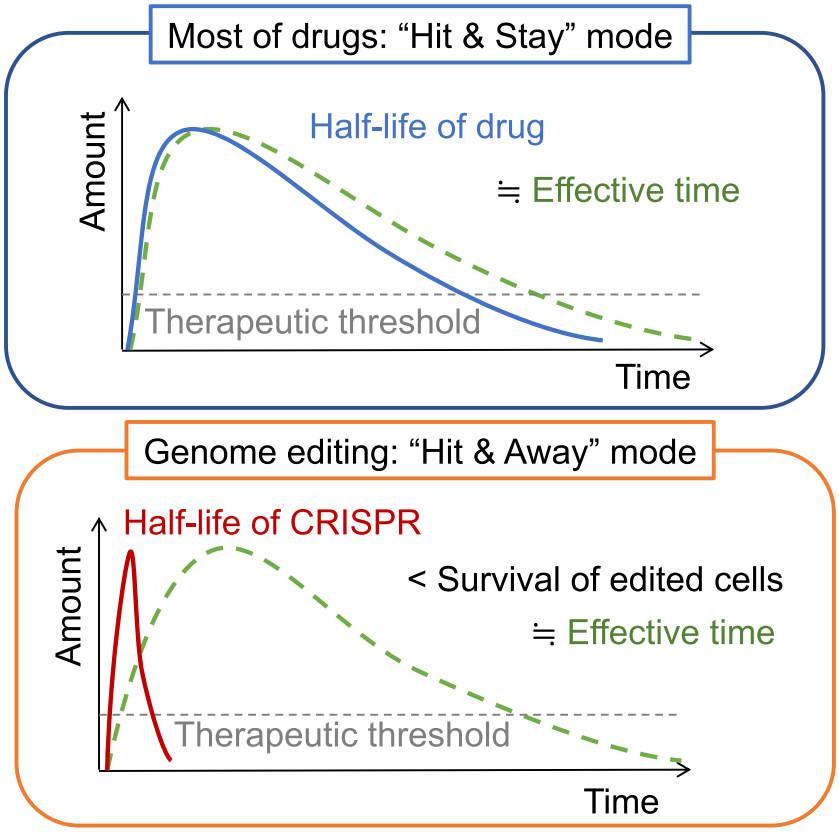

Fig. 3. A conceptual difference of kinetics between conventional drugs and genome editing drugs.
While most drugs are only active while retaining certain concentrations in the body, prolonged stability was the key to achieving a long-lasting therapeutic effect. However, genome editing enzymes can robustly induce genome editing followed by rapid clearance from the body, a feature that is considered favorable to minimize the cellular toxicity, off-target effect, and mutagenesis risk.

Table 3
Summarizes the pros and cons of different biological vectors.

	AAV vector (adeno-associated virus)	AdV vector (adenovirus)	LV vector (lentivirus)	VLP (virus-like particle)
Cargo size limit	< 4.7 kb	8–37 kb	8 kb	> 8 kb?
Duration of expression	Several years in non-dividing cells	< 2 months	Several years	< 1 day
Immunological response	- Neutralization by antibodies.	- Neutralization by antibodies - Cytotoxicity.	- Stimulation of innate immunity. - Antibody production against protein components.	- Antibody production against protein components.
Pros	- High transduction rate. - Derived from a non-pathogenic virus. - A broad range of target cells depends on serotypes. - Extensive experiences in gene therapy. - A small diameter (20 nm) allows better penetration in tissues.	- High transduction rate - Large (8–37 kb) cargo capacity - Minimal genomic integration - A broad range of target cells	- High transduction rate - Large cargo capacity - Less immunogenic. - A broad range of target cells depends on the envelope.	- Transient expression - Simplistic structure and easy to engineer. - No risk of genomic integration - Superior biosafety. - A broad range of target cells depends on the envelope.
Cons	- Pre-existing antibodies preclude the use. - Low or almost no transduction efficiency from 2nd administration due to neutralizing antibody. - Liver toxicity at high dose ($>10^{14}$ v.g./kg). - Integration of the vector genomic sequence. - Prolonged expression (several months to years) leads to increased off-target probability.	- Pre-existing antibodies against Adv. - Low transduction efficiency from 2nd administration due to neutralizing antibody. - Cellular damage.	- Random integration of cargo genome sequence. - Prolonged expression leads to increased off-target probability. - Low transduction efficiency from 2nd treatment due to immunogenicity	- Possible immunogenicity. - No extensive experience in clinical trials (except for vaccine use).

In general, many CPPs contain some basic or cationic amino acid residues, such as arginine (R) or lysine (K). This property is also shared in NLS, as SV40 NLS is composed of “RKKKRKV” amino acid sequence. Strikingly, Doudna’s group has demonstrated that cellular uptake was greatly improved by fusing four SV40 NLSs into Cas9. This finding was further shown by direct injection of NLS-fused RNP into the mouse brain, which resulted in successful genome editing *in vivo* [91]. Direct delivery of NLS-fused Cas9 is advantageous as no additional formulation

is required. Whether this approach can be applicable to tissues other than the brain or larger animals needs further investigation.

Since genome editing occurs within the nucleus, prokaryotic Cas9 protein is usually fused with multiple NLS peptides. Maggio et al. have demonstrated that the AdV delivery of SpCas9 with four NLSs into muscle progenitor cells resulted in significantly higher editing frequencies than the SpCas9 with two NLSs [92]. Therefore, the utilization of NLS is advantageous not only for gene delivery but also for nuclear entry.

Despite the considerable progress with CPP-based delivery in various preclinical studies, there are no CPP-based drugs that have been approved yet by the US Food and Drug Administration (FDA) [93]. Indeed, CPP has several limitations that impede their clinical application. First, CPP is unstable by proteolytic enzymes, and its plasma half-life is relatively short. Thus, CPP may be depredated before being reaching the target tissue. Even after cellular entry, endosomal escape is another barrier to overcome. Second, CPP has little cell-type specificity, which might induce systemic toxicity and limit therapeutic efficiency. More extensive and in-depth research is needed to optimize the CPP for the given cargo and target cell type to accomplish a safe and efficient therapy.

4. Chemical delivery methods

To avoid the risk of immunogenicity against the protein component of the delivery system, the usage of chemical components is a promising approach.

4.1. Gold nanoparticle

Gold nanoparticle (AuNP) or CRISPR-Gold has been demonstrated as a delivery vehicle of Cas9 RNP and donor DNA *in vivo* [94]. The CRISPR-Gold comprises 15 nm AuNP conjugated to thiol modified oligonucleotides, which are hybridized with a single-stranded donor oligonucleotide (ssODN) and complexed with Cas9 RNP and coated with the endosomal disruptive polymer PAsp (DET). The AuNP is internalized by cells *via* endocytosis due to the cationic nature of PAsp (DET). After endocytosis, the PAsp (DET) polymer triggers endosomal disruption and releases Cas9 RNP and donor DNA into the cytoplasm. Co-delivery of Cas9 RNP and a donor DNA result in inducing homology-directed repair (HDR) in skeletal muscle of *mdx* mice, at the efficiency of 5.4% [94].

4.2. Polymer and lipid-based nanoparticle

Lipid-based delivery systems are considered promising non-viral

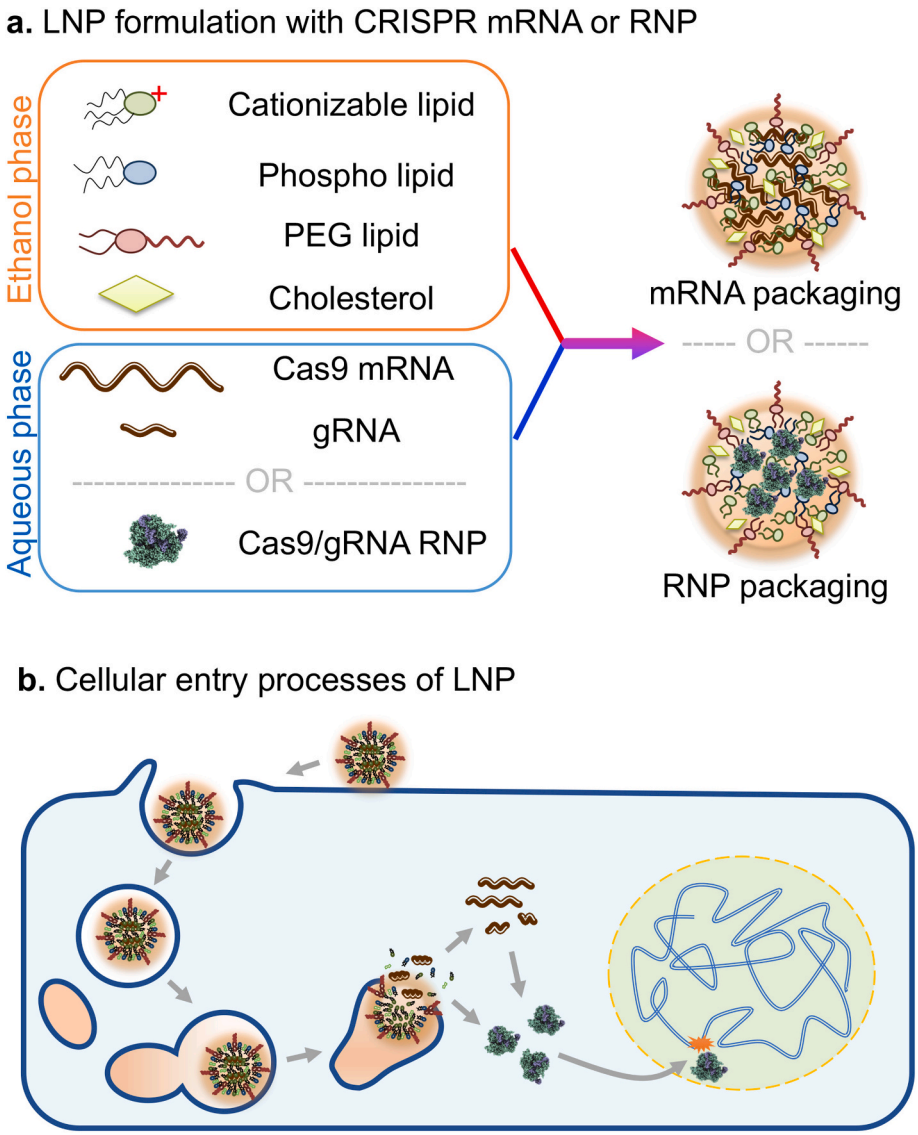

Fig. 4. Schematic representation of the formulation and cellular entry mechanism of lipid nanoparticle (LNP).
a) To formulate LNP encapsulated with RNA or RNP, several lipids in the ethanol phase and RNA/RNP in the aqueous phase are mixed in a microfluidic mixer. Typically, four kinds of lipids are used, but additional lipid (such as cationic lipid) or a molecule (such as a carrier DNA) to help RNP packaging might be added as well.
b) Cellular entry process of LNP. Once LNP is incorporated inside a cell (typically through endocytosis), some LNPs are trapped in endosomes. The acidic condition of the endosomes induces the cationization of the cationizable lipid, particle collapse, and endosomal escape. Cas9 proteins are translated from the released mRNAs, form a complex with gRNA, travel to the nucleus by a nuclear localization signal, and find the target site within the genomic DNA.

vehicles for delivering CRISPR-Cas9 systems. Cationic lipid-based transfection reagents, such as lipofectamine are widely used to transfect cultured cells *in vitro*. However, they are incompetent for *in vivo* use due to the high toxicity of cationic lipid and low transfection efficiency of *in vivo* tissues because of massive interaction with anionic cellular membranes. Therefore, there have been many attempts to modify the lipid-based vectors to provide safer and greater efficiency.

In a study by Abbasi et al., the co-encapsulation of Cas9 mRNA and gRNA in a single polyethylene glycol (PEG)ylated polyplex micelle was shown to prevent the sgRNA release upon dilution, which enhanced the protection of sgRNA from enzymatic degradation. The research group observed that the polyplex micelle allows for the induction of genome editing in the mouse brain [95].

Recently, lipid nanoparticle (LNP) or solid nanoparticle technology is gaining attention, owing to the tremendous success of the mRNA vaccine against the COVID-19 pandemic [96–98]. LNP is one of the most sophisticated delivery technologies composed of typically four kinds of lipids: cationizable (or ionizable) lipid, PEG lipid, helper phospholipid, and cholesterol (Fig. 4a). The key technology of LNP is a pH-dependent cationizable lipid, which is neutral at the delivery stage (neutral pH) but becomes cationic at acidic pHs, such as endosomal entry (Fig. 4b). The change of charge induces the dissociation of particles and disruption of the endosomal membrane to enhance endosomal escape [99]. This pH-dependent property of cationizable lipid overcame the systemic toxicity of cationic lipid. Also, the addition of PEG lipid provides particle stability, shielding from immune recognition and enhancing blood stability. In addition, the helper phospholipid and cholesterol help stabilize the structure of LNP and mediate fusion with the cellular membrane. Since there is no protein or peptide component exposed externally, LNP is considered to be less immunogenic than viral vectors.

As an example of LNP based delivery of CRISPR-Cas9 mRNA and gRNA, Qiu et al. have developed an LNP system to efficiently deliver the CRISPR-Cas9 and gRNA targeting the *Angptl3* gene into the mouse liver [100]. The *Angptl3* gene is an important regulator of lipoprotein metabolism. Silencing or knockout of the *Angptl3* reduces blood lipoprotein levels, suppressing atherosclerosis and coronary heart diseases. Another study also demonstrated that a single injection of LNP packed with CRISPR-Cas9 mRNA and gRNA targeting *Angptl3* disrupted the *Angptl3* gene successfully in mice and reduced low-density lipoprotein cholesterol by 57% and triglyceride by about 29% for over 100 days [101].

LNP was first developed to package RNA molecules, such as siRNA or mRNA, that are negatively charged. Considering the advantage of delivering Cas9 RNP, some researchers tried to package Cas9/gRNA RNP complex, which is rather positively charged. For example, Wei et al. have developed a modified LNP that contains a permanently cationic lipid [102]. The authors reported that the supplemental lipid component mediates encapsulation of RNPs with retention of activity and redirect DNA editing to targeted tissues, including the lung and liver, following intravenous injection in mice. Moreover, the modified LNP delivered Cas9 RNPs into a skeletal muscle to restore dystrophin expression in *mdx* mice [102]. In addition, Suzuki and colleagues used single-strand DNA or RNA as a helper molecule to enhance LNP-based CRISPR-Cas RNP delivery. Finally, they demonstrated significant suppression of hepatitis B virus (HBV), a covalently closed circular DNA (cccDNA) in HBV-infected human liver cells [103].

To expand the tissue targetability, Cheng et al. have reported a strategy termed selective organ targeting (SORT) wherein a supplemental lipid (designated a SORT molecule, such as DOTAP or 18PA) was added on top of the regular LNP formulation. The SORT-LNP was capable of selectively targeting multiple tissues, including liver, lung, spleen, epithelial cells, endothelial cells, B cells, and T cells. Furthermore, the authors demonstrated the compatibility of SORT-LNP for packaging various kinds of cargo, such as luciferase mRNA, Cas9 mRNA/gRNA, and Cas9 RNP [104].

LNP-Cas9 RNP can be combined with the chemoattractant chemokine (C-X-C motif) ligand 12 *(CXCL12α)* and mesenchymal stem cell membrane–coated nanofibril scaffolds mimicking the bone marrow microenvironment for human leukemia stem cells (LSCs) [105]. The *CXCL12α* release induced migration of LSCs to the scaffolds, while the LNP-Cas9 RNP generated the efficient knockout of the interleukin-1 receptor accessory protein (*IL1RAP*) gene, and consequently reduced the colony-forming capacity of LSC and leukemic burden *in vitro* [105]. The primary advantage of combining the scaffold with LNP is to increase the retention time of LNP-Cas9 in the bone marrow cavity, thereby achieving sustained delivery of the genome editing components [105].

With the development of LNP, the first phase 1 clinical trial using LNP-CRISPR was conducted in 2020. A recent publication provides robust results in humans on the efficacy of LNP-based CRISPR-Cas9 mRNA delivery, called NTLA-2001, a therapeutic *in vivo* gene-editing therapeutic agent targeting transthyretin (*TTR*) gene for treating transthyretin amyloidosis (Table 2). Transthyretin amyloidosis is a progressive and fatal disease characterized by progressive accumulation of misfolded TTR protein in multiple tissues, predominantly the nerves and heart [106]. The preclinical studies using mice and cynomolgus monkeys showed a durable knockout of *TTR* in the liver when LNP-CRISPR was injected intravenously. In phase 1 clinical trial led by Intellia Therapeutics, the administration of the LNP-CRISPR NTLA-2001 resulted in a significant reduction (87%) of serum TTR protein concentration after administration of a single dose (0.3 mg/kg) in 3 patients, besides no serious adverse events were reported [106]. The results demonstrate the high potential of LNP for delivering CRISPR-Cas9 into the human liver. Further improvement of LNP is expected to expand the variety of organs and genetic diseases that can be targeted.

Our group recently developed a novel cationizable lipid TLC053 for targeting skeletal muscle tissue to deliver CRISPR-Cas9 mRNA and gRNA [107]. We demonstrated that LNP-CRISPR could be repeatedly administrated to accumulate dystrophin protein recovery in a mouse model, which was not possible with AAV-CRISPR because of clearance by the host immune system. Single intramuscular injection of LNP-CRISPR was sufficient to recover dystrophin protein expression for 12 months in an *mdx* mouse model. In addition, LNP-CRISPR can be administrated through limb perfusion to target multiple muscle groups by a single shot. We believe those properties of LNP-CRISPR could propose a novel approach to treat muscular disorder for treating individual or multiple muscle groups gradually *via* repeated injections.

5. Physical delivery methods

5.1. Electroporation

Electroporation is a physical delivery method based on electric field pulse(s) for promoting the entry of small and macromolecules into a cell. Electroporation has been shown to efficiently deliver Cas9 RNP into hard-to-transfect cells, including iPSCs [8]. For example, to selectively disrupt the *HLA* (human leukocyte antigens) genes to avoid HLA-mediated allogenic reaction by T cells, electroporation has been used to deliver Cas9 RNP/gRNA to generate HLA-edited iPSCs [108]. In addition, Stadtmauer et al. delivered CRISPR-Cas9 RNP by electroporation into human primary T cells to knockout *TCRα* (T cell receptor alpha), *TCRβ*, and *PD-1* (programmed cell death receptor-1) genes and to knock-in a cancer-specific *TCR* transgene (NY-ESO-1) to improve antitumor immunity in three patients with refractory cancer [109]. Furthermore, Xu et al. ablated the *CCR5* (C—C motif chemokine receptor 5) gene (which is a co-receptor of HIV) by electroporation of Cas9 RNP in HSPCs and transplanted into a patient with HIV infection and acute lymphoblastic leukemia [110], as *ex vivo* genome editing therapy.

For *in vivo* use of electroporation, although it is still at the research stage, Kawasaki's group utilized *in utero* electroporation to induce CRISPR-Cas9 for disrupting the *Satb2* gene (which is responsible for callosal axon projections in the developing mouse brain) in mouse brain [111]. It seemed that the clinical application of electroporation is mainly limited to *ex vivo* settings. For *in vivo* genome editing therapy

applications, further optimization of electroporation methods and safety assessments would be necessary. Because the treatable area is limited, targeting a small organ or targeting at a relatively early stage of development (*i.e.*, newborn) might be considered. Important to mention, from an ethical point of view, performing genome editing at the fetus stage or earlier should undergo rigorous oversight by third-party professionals and should adhere to relevant laws and guidelines.

5.2. Microinjection

Microinjection is a method of directly injecting genome editing components inside the cell or nucleus under the microscope. Due to the limited throughput, the zygote stage is the primary target of this delivery method to create a genetically modified animal model for biomedical research [112]. For instance, Gu et al. has established fumarylacetoacetate hydrolase ($FAH^{-/-}$)-deficient pigs *via* cytoplasmic microinjection of the CRISPR-Cas9 system into pig zygotes [113]. The $FAH^{-/-}$-modified pigs were used to model hereditary tyrosinemia type I (HT1) and to identify novel therapeutic approaches for treating this condition [113]. Another recent study utilized the CRISPR-based microinjection strategy to mediate genetic correction of the paired box 6 (*PAX6*) gene, a master regulatory gene for eye development, to reverse vision loss in mouse germline [114].

As a major advantage of microinjection, there is no limitation on the molecular size of the cargo. The injection procedures are typically visualized under the microscope, so the success of cargo delivery can be monitored. On the other hand, microinjection requires specific equipment and technical expert to prevent cell damage. Additionally, only one cell is targeted per injection; therefore, the technique is not convenient when a large number of cells need to be processed. Obviously, therapeutic genome editing in a human zygote should not be performed as therapeutic benefits and safety risks are unproven. Moreover, debate continues regarding the ethics of human embryonic genome editing for potential medicinal purposes.

5.3. Sonoporation

Sonoporation employs the injection of microbubbles and cargo molecules together, followed by applying ultrasound at the target site. By this, microbubbles rupture and generate transient pores on the blood wall and cell membrane that allow the entry of the target cargo into cells [115]. For instance, Ryu et al. applied the ultrasound-activated particles as CRISPR-Cas9 DNA plasmid delivery for disrupting the steroid type II 5-alpha-reductase (*SRD5A2*) gene, which encodes an enzyme involved in male pattern baldness, into dermal papilla cells of a mouse model to recover hair growth [116]. In another study, the ultrasound microbubble-mediated CRISPR-Cas9 plasmid delivery was shown to knockout the epidermal growth factor receptor 2 (*C-erbB-2)* gene, a proto-oncogene associated with breast cancer, in human endometrial cancer (HEC)-1A cells [117].

The physical delivery method alone may not be efficient for *in vivo* delivery, but these methods may be combined with other biological or chemical delivery technologies. Exciting possibilities to explore the combination of multiple delivery methods await future development.

6. Remaining challenges and limitations associated with *in vivo* delivery

As we summarize above, remarkable progress has been made with the delivery technology of CRISPR-Cas9 in advancing therapeutic genome editing *in vivo*. However, there are remaining hurdles and challenges for the successful development of CRISPR-Cas9-mediated therapy. From here, we would like to discuss the limitations and potential future directions related to the safety and efficacy of delivering genome editing tools *in vivo*.

6.1. Repeated administration and immunity against carrier or Cas9

The adult human body is estimated to be consist of 37 trillion (3.72×10^{13}) cells [118]. The necessary treatment area or cell number significantly varies depending on the target organ or tissue, disease type, and severity. For example, when targeting a small organ with a limited cell number, it is relatively easy to achieve a therapeutic threshold by one shot in general. However, large tissues and organs such as skin, liver, lung, or skeletal muscle, consist of substantial cell numbers, and treating such large tissues or organs with a single shot is not realistic with the currently available delivery technologies. Therefore, the ability of repeated administration is a critical feature for targeting disorders that are affected in large tissues/organs (Fig. 5). The delivery technology must be safe enough and not inhibited by the host immune system to enable multiple administrations. For example, as discussed in the above section, AAV is neutralized by an anti-capsid antibody; hence the second injection is not effective [48].

Immunogenicity against CRISPR-Cas9 protein has been discussed as a potential concern for therapeutic applications because more than 50% of the human population possessed preexisting anti-Cas9 antibodies against the most commonly used SaCas9 and SpCas9 bacterial orthologs [119,120]. Chew et al. demonstrated that, regardless of delivery method (AAV9-Cas9 or plasmid-Cas9 electroporation), the expression of Cas9 in mouse muscles significantly elevated the frequencies of $CD45^+$ immune cells and evoked cellular immune responses. While the administration of empty AAV9 without Cas9 did not elicit any significant cellular infiltration, indicating a Cas9-driven immune response [121]. They also demonstrated the absence of extensive cellular damage from AAV-CRISPR-Cas9, whereas a substantial myofiber degeneration was observed in muscles of mice electroporated with plasmid DNA encoding Cas9 [121]. The authors assessed the significant muscle cellular damage observed following DNA electroporation than vehicle electroporated control, suggesting the Cas9 transgene-dependent intramuscular $CD45^+$ cellular infiltration, antibody (IgG and IgM) production, IL-2 and perforin secretion, and myofiber degeneration. Notably, this could be partially reduced by immunosuppression treatment.

To minimize the immunogenicity issue, Cas9 protein can be engineered to remove immunogenic epitopes [122]. Combination with transient immunosuppression is a possible countermeasure for suppressing immunity against not only Cas9 but also delivery cargo. In the case of the direct delivery of Cas9 RNP with CPP, the anti-Cas9 antibody could significantly affect delivery efficiency as antibody binding might mask the CPP part. On the other hand, most other delivery technologies do not expose the Cas9 epitome externally, so the effect of the anti-Cas9 antibody in serum should be minimal, at least during delivery. Encapsulation of the Cas9 in a nanocarrier system might provide temporary protection against neutralizing antibodies and nuclease/protease-mediated degradation [123]. For this, chemical delivery carriers have advantages over biological delivery vehicles. Further study will be needed to address the impact of immunological responses against Cas9.

6.2. Scalability and species difference

When translating genome editing preclinical to human patients, scalability and species difference are also important aspects to consider. For example, the number of cells that need to be transduced to observe clinical significance is roughly 1000 times more in mice than cultured cells in a dish and is about 2000 times more in humans than in mice (Fig. 6). This means that more delivery vectors are needed to be produced to perform human genome editing. Small-scale production of viral or non-viral-based vectors for *in vitro* and mouse is relatively doable in standard laboratory settings. However, mass production at clinical grade requires extra expenses and labor to overcome.

Species difference also needs to be carefully evaluated when moving to human trials. For example, preclinical evaluations in animal models are perhaps the closest representation of what kind of result we would

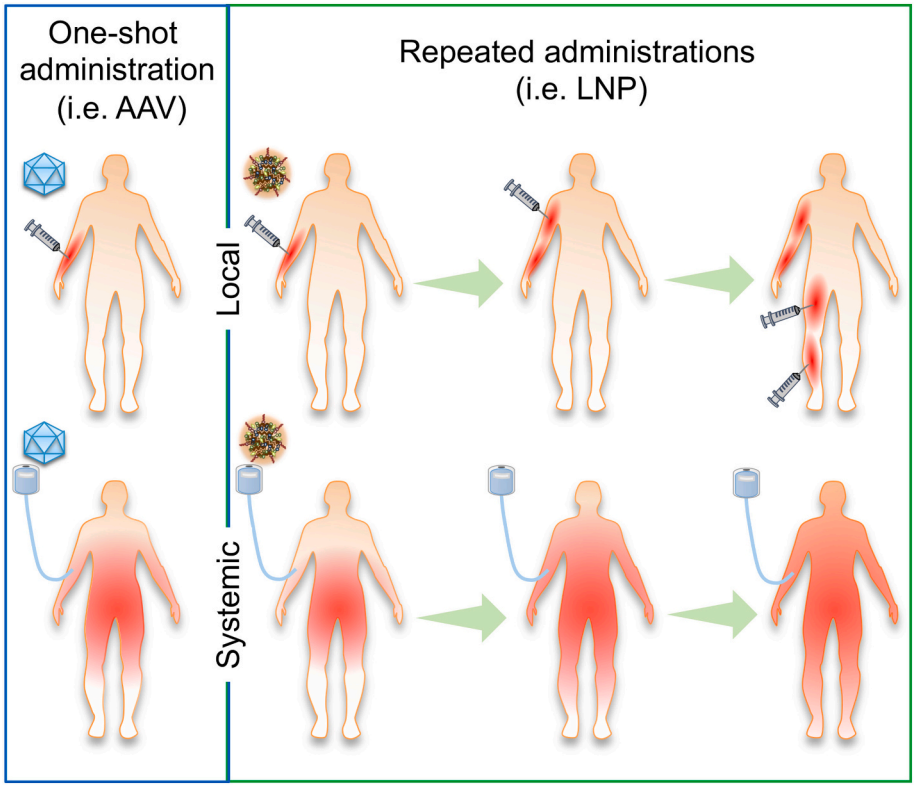

Fig. 5. Comparison of one-shot administration and repeated administration.
The number of possible administrations highly impacts the treatable area for genome editing therapy. For local injection, repeated dosing allows expansion of the treatable area over time. For systemic infusion, not only the treatable area but also the efficacy might be accumulated and enhanced by multiple dosing. Important to note, the concept of repeated dosing is applicable only when the genome editing effects accumulate for a sufficient amount of time.

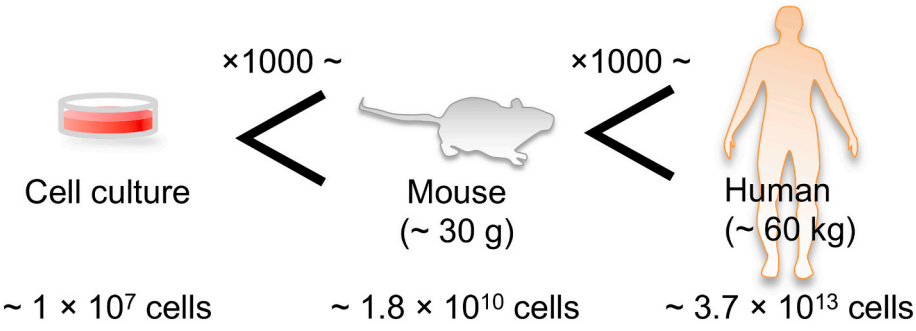

Fig. 6. Schematic illustration of research transition from *ex vivo* testing, *in vivo* validation in a mouse preclinical model, and final application for a human clinical trial.
For *ex vivo* genome editing, cells are directly exposed to the culture environment, and only ~1×10^7 cells need to be transduced for a 10 cm culture dish. On the other hand, when we move to *in vivo* pre-clinical or clinical studies, it is vital to ensure the delivery methods is scalable to transduce thousand-fold more cell number in a mouse model (~ 1.8×10^{10} cells/for a mouse 30 g) or the human body (~ 3.7×10^{13} cells/ for a person with 60 kg). Important to add, a mouse is not a tiny human, so the difference in species needs to be considered as well. Genome editing requires targeting a specific DNA sequence, so targeting a common sequence between mouse and human is a reasonable approach, if applicable. Otherwise, creating either a transgenic mouse by inserting the human target sequence or a humanized mouse by replacing the mouse sequence with the human one would be important to assess genome editing activity in a mouse context.

expect when performed in humans. However, differences in body structure, metabolic state, or gene expression profile indicate that clinical translation should be carefully assessed before being tested on human patients [124,125]. Since CRISPR-Cas9 works in a target sequence-dependent manner, targeting a common DNA sequence between animal models and humans would be important to consider. If this is not feasible due to the sequence limitation, creating a humanized animal model with the human target sequence could be an option. For instance, cultured human cells or organoids differentiated from iPS cells can be utilized for assessing *in vitro* efficacy and off-target risk.

6.3. Mutation type and low HDR compared with NHEJ in vivo

Once CRISPR-Cas9 is delivered into a cell, Cas9 creates a double strand-break (DSB) at the target DNA, and the site undergoes one of the major repair pathways, either non-homologous end joining (NHEJ) or homology-directed repair (HDR) [126]. NHEJ involves direct ligation of the two DSB ends with no homology overhangs, which possesses a high risk of indel error. The HDR pathway, on the other hand, requires a homologous DNA template and can create accurate deletions, insertions, or substitutions [126]. Considering that the majority of pathogenic genetic variants are caused by single-nucleotide variants [127], the HDR pathway is highly preferred for correcting such genetic mutation or for inserting a transgene in a site-specific manner. However, most cells in our body are arrested in the G0/G1 phase of the cell cycle [128]. This is a significant hurdle for HDR-mediated *in vivo* genome editing because HDR is limited to the S/G2 phase while NHEJ is active throughout the cell cycle [129,130]. To overcome this issue, some groups tested NHEJ inhibitors (*i.e.*, DNA ligase IV inhibitor Scr7) or HDR enhancers to increase the probability of cells proceeding through HDR in cell lines and mouse zygotes [131]. Another option in cell culture is cell cycle synchronization [132]. However, these methods are not feasible for *in vivo*

genome editing because the acceleration of the cell cycle poses potential cytotoxic or tumorigenic risks.

Apart from the cell cycle, HDR requires a DNA donor template. Single-stranded DNA (ssDNA) donor is becoming the preferred template because it has shown superior efficiency compared to dsDNA template with lesser risk of genomic integration. For *in vivo* genome editing *via* HDR, recently, Siegwart et al. developed an all-in-one dendrimer-based lipid nanoparticle to package Cas9 mRNA, sgRNA, and donor ssDNA. By doing so, they achieved HDR efficiency of approximately 56% *in vitro* and 20% in xenograft tumors in mice [133].

Engineering Cas9 enzyme to enhance HDR efficiency was also attempted. Charpentier et al. tried fusing CtIP (RBBP8) protein, an essential protein for HDR initiation, to Cas9 for increasing HDR efficiency in different cell lines and rat zygotes [134]. This enabled artificially anchoring CtIP to the DSB site and increased HDR efficiency ~2 folds higher than Cas9 alone. While this study provides new insight and prospects for using DNA repair proteins with Cas9 to enhance HDR, delivery methods and risk management need to be considered for *in vivo* applications. The minimal CtIP domain required for sufficient HDR stimulation was 296 amino acids (888 bp) in size and roughly 5 kb in total, including Cas9. Hence, it will be difficult to package inside commonly used delivery vectors such as AAV vectors. Furthermore, when the donor template was not given, Cas9-CtIP seemed to result in different indel patterns compared to Cas9, posing a risk of unexpected edits. Most indels were deletions flanked by microhomologies. This is likely due to forcing the DNA resection pathway by CtIP. Therefore, when artificially altering global DNA repair pathways by overexpression, all possible outcomes and potential risks should be carefully assessed in advance.

The liver is one of the popular targets for genome editing because of its regeneration capacity, which not all organs have. Previous studies showed that the combination of zinc-finger nucleases and ssDNA could induce high HDR efficiency *in vivo* through AAV delivery into the liver [135–137]. This was possible due to the high transduction efficiency of AAV and the nature of ssDNA, the genome of AAV, as a good HDR template.

Hopefully, more techniques will come out in the future to help enhance HDR editing efficiency *in vivo* so that single nucleotide alterations or targeted integration can happen in various organs. Instead of challenging HDR *in vivo*, alteration of a single nucleotide might be induced by novel genome editing tools (such as a base editor or prime editor, as discussed in the below section) or targeted integration systems like the homology-independent targeted insertion (HITI) method [138].

6.4. Genomic integrity, off-target risk, on-target unwanted editing

One of the biggest concerns associated with genome editing therapy would be the risk of off-target mutagenesis. The degree of risk also depends on the level and duration of Cas9/gRNA expression [139]. Thus, from a delivery perspective, transient expression is better to reduce the risk of off-target mutagenesis than long-term expression (such as AAV). Moreover, the possibility of AAV-Cas9 genomic DNA integration was reported, which may lead to unwanted genome alterations or genotoxicity [55,56]. To avoid DSB associated with CRISPR-Cas9, researchers have started to develop alternative tools. These include base editors, prime editors, and epigenetic editors. Below we introduce the advanced type of genome editing tools to discuss the possibility for clinical application and challenges associated with delivery methods.

7. Recent advances in genome editing tools

It has been almost a decade since Cas9 was first discovered to be harnessed for genome editing in 2012 [1]. Since then, the variety of genome editing tools has expanded in which some cases might be more efficient and specific than the conventional Cas9 system, without relying on the stochastic DNA repair pathways of DSB. These new editing systems provide a powerful toolset for the potential treatment of complex genetic diseases. However, they typically require a larger cargo size and undergo a different editing mechanism. In order for these new tools to be applied for therapeutic usage, further optimization of its efficiency and safety is required as well as a suitable delivery method to produce maximal editing efficiency *in vivo*. Here we summarize recent progress for base editors, prime editors, and epigenetic editors for *in vivo* application and limitations to overcome.

7.1. Base editor

Base editors were developed to introduce or alter single nucleotide variations. By fusing dead Cas9 (dCas9) or nickase Cas9 (nCas9) with the deaminase domain, base editors allow editing of single nucleotides through direct conversion of the target nucleobase. Cytosine base editors (CBEs) convert C•G to T•A base pairs by converting cytidine to uridine leading to C➔T (or G➔A) substitution [140]. This is achieved by fusing a cytidine deaminase enzyme with nickase Cas9 tethered to one (BE3) or two (BE4) monomers of uracil glycosylase inhibitor (UGI). Similarly, adenine base editors (ABEs) create an A•T to G•C substitution by utilizing an adenine deaminase [141].

The two main advantages of base editors are that they do not induce DSBs and do not require donor DNA templates to introduce nucleotide change [140,141]. Already, numerous reports demonstrate the successful base editing in mice and zebrafish using both CBEs and ABEs [142–144]. But before base editors can be applied for *in vivo* therapeutic usage, its safety matters must also be considered.

Despite their usefulness, CBEs and ABEs suffer from unwanted on-target bystander edits and off-target effects. For CBEs, unwanted base conversions are possible when multiple “C”s are present near or within the target window. This unwanted on-target editing is so-called bystander activity. Moreover, recent reports also showed unwanted deamination of bystander cytosines when using ABEs [145]. To overcome this issue, efforts have been made to narrow the editing window and engineer the deaminase enzyme to produce less bystander edit and off-target effect; however, they may suffer from a cost of editing efficiency [145–148].

sgRNA dependent and independent off-target are also a major concern for CBEs and ABEs. Genome-wide profiling of base editors using Digenome-seq and EndoV-seq revealed sgRNA dependent off-targets [149–151]. Although gRNA-dependent off-target mutagenesis with base editors are much more infrequent compared to wild-type Cas9, there is a need for a more thorough analysis. For example, a recent study led by Chengqi Yi evaluated genome-wide off-target edits using Detect-seq, which utilizes chemical labeling and biotin pulldown to trace intermediate deoxyuridine created by CBEs [152]. CBEs are supposed to edit only the protospacer adjacent motif (PAM)-containing strand. However, Detect-seq detected unexpected edits outside the protospacer sequence not so far from the sgRNA site and on the target strand dependent and independent of Cas9/gRNA. The mechanism for such off-target effects of CBEs is unknown, but the fused deaminase domain might function at a slightly distal site from the gRNA binding site. Further research will be necessary to address this issue.

The sgRNA independent off-target is thought to be caused by the intrinsic DNA affinity of the deaminase domain and its attachment with dCas9 [153]. Two groups reported off-target effects of CBEs using BE3 even without the presence of sgRNAs [154,155]. Most of the sgRNA independent off-target editing is located at transcription sites, raising safety concerns by altering the transcriptional profile. However, genome-wide sgRNA-independent deamination with ABEs seems to be lower compared to CBEs [155]. Many studies also report prevalent sgRNA independent RNA deamination in different base editors. For example, a study performing RNA-seq showed substantial transcriptome-wide deamination of RNAs [149]. CBEs were reported to induce tens of thousands of C to U edits in both protein-coding and non-protein-coding RNA sequences. The same phenomena were seen with

ABEs where adenine deamination was detected in RNAs. However, the consequences of RNA deamination and its clinical impact are not yet fully evaluated because of the short lifespan of RNA molecules. So long as the expression profile of the base editor is transient, the effect of RNA editing should also be temporary.

By delivering ABEs through the LNP system, two groups were able to target the proprotein convertase subtilisin/kexin 9 (*PCSK9*) gene and reduce low-density lipoproteins (LDL) cholesterol levels in macaques [156,157]. Editing efficiency over 60% was achieved in liver hepatocytes and reduced LDL cholesterol levels up to 8 months. In addition, CIRCLE-seq and CHANGE-seq analysis detected no significant gRNA independent or dependent off-target effects in the genome. These results support effective and safe adenine base editing *in vivo* and potential therapeutic usage for genetic liver diseases.

7.2. Prime editor

Prime editing is a highly flexible editing tool that can potentially make a single base substitution or small deletions and insertions near the Cas9/gRNA target site. Along with the development of base editors, Liu's group also created a new CRISPR genome editing tool that can make single, small, and medium-range changes called prime editing [158]. Prime editors consist of a prime editing guide RNA (pegRNA) and a MoMLV (Moloney murine leukemia virus) reverse transcriptase that is fused to Cas9 (H840A) nickase. The pegRNA is an extended sgRNA that contains the guide sequence and the reverse transcriptase template sequence. Once H840A nicks the non-complementary strand of the DNA, it exposes the 3′-hydroxyl group, allowing the reverse transcriptase template binding and initiating reverse transcription. This creates an intermediate of two DNA structures, a 3′ flap having the edited sequence and the 3′ flap containing the unedited sequence. However, endogenous endonucleases excising the 5′ flap make the hybridization of the 3′ flap thermodynamically more favorable, creating a heteroduplex. If needed, the newer version of prime editing systems PE3 and PE3b facilitated the incorporation of the edited strand by introducing a nick in the unedited strand by an additional sgRNA.

Prime editors provide two main advantages over the Cas9 editing system. First, prime editing does not induce any DSB because it uses nickase. Second, prime editing does not rely on the HDR DNA repair machinery. A study conducted by Miano's group directly compared the efficiency for prime editing and HDR editing with Cas9 in mice [159]. Results showed 55% of the sequences with correct editing using Cas9 and ~ 21% using prime editing. But, for Cas9, the amount of on-target bystander indels (~40%) and off-target edits were much higher, while no indels and off-targets were found with prime editing. The third advantage of prime editing is its wide range of possible modifications. With the current base editors developed so far, only 4 out of the 12 possible nucleotide changes are possible. For prime editing, not only all 12 possible base editing combinations but also small insertions or deletions are possible to edit. While base editors suffer from bystander effects at a detectable level, prime editors result in very few bystander editing effects. Recently, Kweon et al. successfully engineered different SpCas9 variants used for PE2 and altered the PAM specificity to expand the number of targetable sequences using prime editor [160]. Prime editing is a relatively new technique compared to the other genome editing tools, and more optimization is required [161,162]. Many groups report the successful prime editing for *in vitro*, organoid, and *in vivo* genome editing [163,164]. But the editing efficiency seems to vary largely depending on the cell type, target locus, and experimental condition. Further understanding of the mismatch repair pathway and engineering of primer editor/pegRNA will be vital to achieving higher editing efficiencies.

Regarding clinical viability, more testing and optimization will be needed on different cell types. The delivery method of prime editors *in vivo* is also problematic because the size of the prime editor is relatively large, which is ~6.3 kb (Fig. 2). To deliver prime editor by an AAV vector, Huang et al. constructed a split-prime editor that can be delivered through a dual-AAV vector and demonstrated successful editing *in vitro* and *in vivo* [165]. However, similar to other groups, the editing efficiency *in vivo* when targeting adult mouse retina was low (1.87%) [166]. In addition, since prime editor utilizes the reverse transcriptase domain and nickase domain, risks of generating DNA from RNA and chromosomal integration need to be investigated.

7.3. Epigenome editor

Apart from direct editing of the genetic sequence, epigenetic editing involves modifying the epigenetic status of a specific locus for changing gene expression. This is achieved by typically fusing dCas9 with epigenetic modifiers (Fig. 2). Such modifier changes DNA methylation, histone modification, or chromatin looping, which may ultimately alter gene expression [167,168]. Similarly, transcriptional regulators (activators and repressors) are also used with dCas9 to change gene expression [169–171].

Strictly speaking, epigenome editing is not included in the definition of genome editing therapy, but epigenetic editing provides two main advantages over genome editing tools. Firstly, gene expression can be altered without changing the genomic sequence; hence it does not fall under the category of genetic recombination. Secondly, the effects caused by epigenetic modifiers or transcriptional regulators may be reversed back by removing them [172]. Thus, epigenetic editing is considered to be safer than genome editing tools in principle. Especially, epigenome editors might treat haploinsufficient diseases by enhancing the gene expression of the unmutated allele to compensate for the deficient gene. Already numerous studies report successful epigenetic editing in various organs *in vivo* in mice [173–175]. For example, haploinsufficiency of the *C11orf46* gene leads to hyperexpression of the semaphorin-6A *(SEMA6A*) gene and is associated with disrupted transcallosal connectivity in the brain. C11orf46 is a nuclear protein that recruits repressive chromatin regulators. Kamiya et al. created a *C11orf46* knockdown mouse model to mimic transcallosal dysconnectivity [173]. They then delivered dCas9-SunTag and scFv-fused C11orf46 protein to anchor the multiple C11orf46 proteins at the *Sema6a* related gene promoters by *in utero* electroporation in mice. *Sema6a* expression level was rescued and restored transcallosal connectivity *in vivo*. Another study conducted by Colasante et al. rescued epileptic encephalopathy phenotype in the Davet syndrome mouse model by enhancing *Scn1a* gene expression using transcriptional activator VP160 to overexpress the allele that was still active [174]. Dual AAV9 vector was chosen for *Scn1a*-dCas9A delivery in mice because of its large cargo size and high transduction efficiency in large brain areas.

Many efforts have been made to dissect the function of different epigenetic marks and their relationship with varying expressions of a gene. But it is still not easy to predict every outcome of epigenetic changes on gene expression. Hence, the risk of epigenetic modification on the target gene and surrounding genes should be assessed carefully. Moreover, the effect of chromatin modification seems to depend on organism, cell type, locus, and developmental stage [176–178]. How to assess potential health risks caused by unexpected epigenome changes need to be established.

Ensuring that stable epigenetic modifications are inherited to their daughter cells is also an essential factor to consider. How cells specifically retain epigenetic memory is still a matter of intense investigation and is context/gene dependent [178,179]. When performing epigenetic editing, selecting the appropriate tool and targeting site is essential for retaining long-lasting epigenetic memory. For example, in a study that compared Kruppel-associated box (KRAB) induced silencing and DNA methyltransferase 3A (DNMT3A*)* induced methylation, the effects from KRAB alone were quickly reversed while when combined with DNMT3A-methylase domain, the maintenance of gene silencing was significantly longer in cultured cells [172].

In addition, utilizing an appropriate delivery vector is also essential

for success in epigenetic editing. With epigenetic editors, too short expression by a transient delivery might not be enough to produce heritable changes. Though context-dependent, whether brief or prolonged expression of the epigenetic modifier is sufficient to observe phenotypic significance should also be assessed. Epigenetic editing holds a prospect in treating epigenetic-related diseases and other genetic diseases like sickle cell anemia and muscular dystrophy by the activation of a compensating gene [180]. Further understanding of epigenetic mechanisms will open possibilities of epigenome editing therapy in targeting more complex diseases such as genomic imprinting or X-chromosome inactivation.

Which genome editing tool to use will highly depend on disease type, target sequence, mutation type, correction strategy, and epigenetic state. Each editing tool has its own advantages and disadvantages, while some still need more optimization and development. However, these new tools face a common problem: they need a large delivery vehicle that is big enough to carry all the necessary components. The development of such a vector will help move other recent genome editing tools toward clinical application. Apart from delivery vector, the use of smaller Cas orthologs for base editors or other editing domains may provide a solution to reducing cargo size. Nevertheless, further optimization and finding an appropriate delivery method will be crucial for successfully developing base editing therapy, prime editing therapy, or epigenome editing therapy *in vivo*.

8. Conclusion

The discovery and development of CRISPR-Cas9 and other recent genome-editing tools have made genome editing viable for treating multiple diseases. Although the CRISPR-Cas systems have shown promising results in cell culture and animal models, the clinical translation of CRISPR-based therapies remains challenging by the absence of safe and effective methods to deliver the genome-editing components into broad tissues and organs, while avoiding immunogenicity and risk of genotoxicity. Further development and improvement of the delivery technology will open infinite possibilities for genome editing therapy.

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