Review article

Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications

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ABSTRACT

The CRISPR-Cas9 genome-editing system is a part of the adaptive immune system in archaea and bacteria to defend against invasive nucleic acids from phages and plasmids. The single guide RNA (sgRNA) of the system recognizes its target sequence in the genome, and the Cas9 nuclease of the system acts as a pair of scissors to cleave the double strands of DNA. Since its discovery, CRISPR-Cas9 has become the most robust platform for genome editing in eukaryotic cells. Recently, the CRISPR-Cas9 system has triggered enormous interest in therapeutic applications. CRISPR-Cas9 can be applied to correct disease-causing gene mutations or engineer T cells for cancer immunotherapy. The first clinical trial using the CRISPR-Cas9 technology was conducted in 2016. Despite the great promise of the CRISPR-Cas9 technology, several challenges remain to be tackled before its successful applications for human patients. The greatest challenge is the safe and efficient delivery of the CRISPR-Cas9 genome-editing system to target cells in human body. In this review, we will introduce the molecular mechanism and different strategies to edit genes using the CRISPR-Cas9 system. We will then highlight the current systems that have been developed to deliver CRISPR-Cas9 into vitro and in vivo for various therapeutic purposes.

1. Introduction

The clustered regularly interspaced short palindromic repeats (CRISPRs) are repeated segments of DNA originally discovered in prokaryotic organisms. CRISPR and CRISPR-associated (Cas) proteins are a part of the adaptive immune system in archaea and bacteria to defend against invasive nucleic acids from plasmids and phages. The CRISPR structure was first reported by Ishino in 1987 [1], and the acronym CRISPR was proposed by Jansen in 2002 after several similar structures were identified in different bacteria and archaea [2,3]. A milestone happened in 2005 when hyper-variable spacers with sequence homology to foreign plasmids and viruses were discovered. Mojica and colleagues thereafter speculated that the CRISPR structure and its related protein might possess immune defense functions and play significant roles in protecting against transmissible genetic elements [4].

Since then, more details about the CRISPR system have been elucidated at an accelerated pace. Charpentier, Doudna and Zhang are the three crucial contributors to this field. Charpentier was the first to elucidate the mechanism of the CRISPR-associated protein 9 (Cas9) genome-editing system [5]. In addition, Charpentier and Doudna reported the biochemical characterization of Cas9-mediated gene editing and optimized the system [6]. Zhang was the first to adopt the CRISPR-Cas9 system in eukaryotic cells for genome editing [7]. In 2015, CRISPR-Cas9 was named as the “Breakthrough of The Year” by the Science magazine [8].

Cas proteins are endonucleases that use a single guide RNA (sgRNA) to form complementary base pairs with target DNA and then cleave the DNA at specific sites. Among the different types of Cas proteins, Cas9 is the most widely used type because of its simplicity, high efficacy, and ease to use. The Cas9/sgRNA two-component system is highly efficient and specific in gene-editing. sgRNA recognizes a specific sequence in the genome, and the Cas9 protein subsequently acts as a pair of scissors to cleave the DNA sequence. Theoretically, the system can be exploited to engineer almost any DNA sequences in the genome, thus making the CRISPR-Cas9 system the most powerful gene-editing tool so far. One important application of this technology is to quickly generate knockout cell lines or animal models. The CRISPR-Cas9 system has therefore triggered a global research boom in both academia and industry. As shown in Fig. 1, the number of publications about CRISPR-Cas9 has been exponentially increased over the past few years. Its applications have been extended to a great variety of fields, including biological research [9], human medicine [10], biotechnology [11] and agriculture [12].

Although the CRISPR-Cas9 system is a newly developed gene-
In the adaptation stage, foreign DNA fragments (approximately 30–45 nucleotides, also named protospacers) from invading plasmids or viruses are incorporated as new spacers into CRISPR arrays. The selection of protospacers from the foreign DNA is based on the protospacer-adjacent motif (PAM). New spacers then provide sequence-specific memory against their corresponding invading plasmids or viruses [22, 23]. In the expression stage, the CRISPR array is transcribed to pre-CRISPR RNA (pre-crRNA), which is further processed to mature CRISPR RNA (crRNA). Each crRNA contains a conserved repeat sequence and a transcribed spacer, which is complementary to the foreign DNA. A pool of crRNAs can target multiple gene elements because each crRNA corresponds to an invasion sequence [24]. In the interference stage, crRNAs act as a guide to specifically target the PAM, and Cas9 cleaves the matched DNA. In the type II CRISPR-Cas9 system, the sgRNA-Cas9 complex binds to its target DNA to ensure that the Cas9 cuts both strands of the DNA, thus blocking the propagation of foreign DNA [25].

Type II CRISPR-Cas9 is the most routinely used CRISPR gene-editing system and is usually refer to as CRISPR. Scientists have demonstrated how to successfully engineer type II CRISPR system to edit genome in mammalian cells. The Cas9 protein is an endonuclease containing two nuclease domains, RuvC and HNH. The RuvC domain cleaves non-complementary DNA strands, while the HNH domain cleaves complementary DNA strands (Fig. 2A). The sgRNA is composed of the trans-activating crRNA (tracrRNA) and crRNA (Fig. 2B). The crRNA contains a 20-nt protospacer element and an additional sequence that is complementary to the tracrRNA. The tracrRNA hybridizes to the crRNA and binds the Cas9 protein, forming the CRISPR-Cas9/sgRNA complex to create double-stranded breaks (DSBs) at target sites in the genome. The dual-tracrRNA:crRNA is normally engineered as a single-strand sgRNA containing two crucial segments: a duplex RNA structure at the 5′ end to bind Cas9 and a guide sequence at the 5′ end to bind target DNA sequence. As shown in Fig. 2, this two-component system is simple but powerful. sgRNA recognizes a specific sequence in the genome, and Cas9 acts as a pair of scissors to cleave the DNA sequence.

The molecular mechanisms of the CRISPR-Cas9 system-mediated genome-editing are illustrated in Fig. 3. Cas9 protein cuts 3′-nt upstream of the PAM site. After DSBs are formed, either the Non-Homologous End Joining (NHEJ) repair pathway or the Homology Directed Repair (HDR) pathway will be initiated. The NHEJ repair pathway often leads to the generation of insertion/deletion (InDel), leading to frameshifts and/or premature stop codons within the open reading frames (ORFs) of target genes. By contrast, a donor DNA template is needed to repair the DSBs in the HDR pathway. Correct DNA sequences are precisely inserted into the target site using a donor DNA template. The HDR pathway is substantially less effective than the NHEJ pathway because gene replacement or knock-in is usually less efficient than gene knock-out.

CRISPR-Cas9 holds great promise in the therapy of genetic disorders by directly editing disease-related mutations. Tremendous efforts have been devoted to improve the specificity, gene-editing efficacy, and delivery efficiency of the CRISPR-Cas9 system. As a result, CRISPR-Cas9 has become a revolutionary genome-editing tool for a wide variety of therapeutic applications.

**Fig. 2.** Schematic diagram of the Cas9 protein (A) and sgRNA (B). Cas9 protein contains two nuclease domains, the RuvC domain and the HNH domain. The RuvC domain cleaves non-complementary DNA strands, and the HNH domain cleaves complementary DNA strands. The sgRNA is composed of the trans-activating crRNA (tracrRNA) and crRNA. The crRNA contains a 20-nt protospacer element and an additional nucleotides that are complementary to the tracrRNA. The tracrRNA hybridizes to the crRNA and binds to the Cas9 protein, forming the CRISPR-Cas9/sgRNA complex to edit genome sequences.
3. Different strategies to edit genes using CRISPR-Cas9

As illustrated in Fig. 4, there are three strategies to edit genome using CRISPR-Cas9. The first and the most straightforward approach is to use a plasmid-based CRISPR-Cas9 system encoding the Cas9 protein and sgRNA from the same vector, thus avoiding multiple transfections of different components [26]. The second strategy is to deliver the mixture of the Cas9 mRNA and the sgRNA [27]. The third strategy is to deliver the mixture of the Cas9 protein and the sgRNA [28].

The plasmid-based CRISPR-Cas9 system is a simple and convenient strategy that avoids the transfection of multiple components to the same cells. In addition, the plasmid-based CRISPR-Cas9 system exhibits greater stability than the system that combines the Cas9 mRNA with sgRNA. For instance, in the pX260 or pX330 system, Cas9 protein and sgRNA were expressed from the same plasmid. The pX260 system, also known as the pX334 system, contains three cassettes, including a CRISPR RNA array, tracrRNA, and S. pyogenes Cas9 (or the Cas9 D10A nickase). The plasmid is digested with a restriction enzyme and then ligated with an annealed oligonucleotide that is designed for a specific targeting site. Another advanced system is called pX330 or pX335.

Fig. 3. Molecular mechanism of the CRISPR-Cas9 system. Cas9 protein cuts 3–4 nt upstream of the PAM site. After DSBs are formed, either the Non-Homologous End Joining (NHEJ) repair pathway or the Homology Directed Repair (HDR) pathway is initiated. The NHEJ repair pathway often leads to the generation of insertion/deletion (InDel), leading to frameshifts and/or premature stop codons within the open reading frames (ORFs) of target genes. By contrast, a donor DNA template is needed to repair the DSBs in the HDR pathway. Correct DNA sequences are precisely inserted into the target site using a donor DNA template. The HDR pathway is substantially less effective than the NHEJ pathway because gene replacement or knock-in is usually less efficient than gene knock-out.

Fig. 4. Different strategies to edit genes using the CRISPR-Cas9 system. The first and the most straightforward approach is to use a plasmid-based CRISPR-Cas9 system encoding the Cas9 protein and sgRNA from the same vector, thus avoiding multiple transfections of different components. The vector will express the Cas9 protein and sgRNA, which will form the Cas9/sgRNA complex inside cells to edit genomic sequences. The second strategy is to deliver the mixture of the Cas9 mRNA and sgRNA. The Cas9 mRNA will be translated to Cas9 protein in cells to form the Cas9/sgRNA complex. The third strategy is to directly deliver the Cas9/sgRNA complex into cells.
which only contains two cassettes, a chimeric gRNA containing tracrRNA and S. pyogenes Cas9. Similarly, pX330 or pX335 vectors are also digested with restriction enzymes and used for ligation with annealed oligonucleotides that are designed for a specific targeting site [26]. However, the plasmid-based system faces several challenges. First, the plasmid must be delivered into the nucleus, which is generally difficult. Secondly, the plasmid needs to be translated into Cas9 mRNA inside the cells, requiring a long time to edit its target. On the other hand, delivery of the plasmid-based CRISPR-Cas9 systems produces more off-target effects. For example, delivery of a plasmid-based CRISPR-Cas9 system generated small insertions and large insertions in off-target sites [29,30].

Direct delivery of the Cas9 mRNA and sgRNA into target cells edits genome after expressing the Cas9 protein and subsequently forming the Cas9/sgRNA complex inside the cells [9,31]. The advantage of administering mRNAs is the transient expression of Cas9 protein, which limits the duration of gene-editing. In addition, delivery of mRNAs has lower off-target effects than the delivery of plasmid-based CRISPR-Cas9 systems. Apart from subsiding off-target effects, mRNAs only need to enter the cytoplasm to exert their effects. Furthermore, the use of the mRNA encoding Cas9 protein shows low cytotoxicity in primary cells and cell lines [32]. However, the relatively poor stability of mRNA is an obstacle for this type of gene-editing strategy.

Direct delivery of the Cas9 protein complexed with sgRNA is the most widely studied strategy in recent years. Purified Cas9 protein is positively charged and can efficiently form a complex with sgRNA, which is called Cas9/sgRNA ribonucleoprotein complexes (RNPs). Direct delivery of RNPs has numerous advantages, including rapid action; high gene editing efficiency; no requirement of codon optimization and promoter selection; and reduced off-target effects, toxicity and immune responses [33]. Various delivery systems that have been explored for different types of CRISPR-Cas9 are summarized in Table 1.

4. Physical and non-viral delivery of CRISPR-Cas9

In the pioneering stage of applications of the CRISPR-Cas9 system, various physical and non-viral delivery approaches (Fig. 5), such as electroporation [34], nanoparticles [28] and hydrodynamic injection [35] have been used to deliver CRISPR-Cas9 to target cells. Although viral vectors are more efficiently to deliver nucleic acids, such as the plasmid-based CRISPR/Cas9, safety is the primary advantage of non-viral vectors. The lack of a size limitation for transgenic DNA is another advantage of non-viral delivery systems. Importantly, the availability and cost-effectiveness of non-viral delivery systems make them attractive for applying CRISPR-Cas9 to human patients rather than viral delivery systems [32,36,37]. Advantages and disadvantages of these approaches are summarized in Table 2. The commonly used physical and non-viral delivery approaches are discussed below.

4.1. Electroporation

Electroporation is a widely used approach to deliver proteins and nucleic acids into mammalian cells [38]. The permeability of cell membrane is temporarily increased during electroporation, allowing proteins or nucleic acids to enter the cells [39]. Electroporation is suitable for all types of CRISPR-Cas9 systems, including plasmid-based CRISPR-Cas9 systems, the mixture of Cas9 mRNA and sgRNA, and the Cas9/sgRNA RNPs. The limitation of electroporation is that plasmid DNA is only integrated into approximately 0.01% of the target cells. In addition, electroporation induces significant cell death.

Gene editing studies of vertebrate organogenesis have widely utilized the electroporation of plasmid-based CRISPR-Cas9 systems to access zebrafish fin regeneration, axolotl regeneration in embryonic cells, chicken development, and mouse brain development [40–42]. In addition, electroporation has recently been used to deliver plasmid-based CRISPR-Cas9 to cancer cells, CD4+ T cells, CD34+ stem cells, and embryonic stem cells [43–47].

The Cas9 mRNA and sgRNA have also been introduced into cells using electroporation. For example, electroporation was used to deliver the Cas9 mRNA, sgRNA, and donor DNA to zygotes to generate a mouse model with mutated genes [34]. Furthermore, electroporation has been used to deliver RNPs to fibroblasts, embryonic stem cells [33] and CD4+ human T cell [14].

It was reported that electroporation of RNPs yields higher gene-editing efficiency than electroporation of a plasmid-based CRISPR-Cas9 system in axolotl spinal cord cells [48]. Similarly, Liang and colleagues compared the plasmid-based CRISPR-Cas9 system, Cas9 mRNA/sgRNA and RNPs. Electroporation of RNPs into certain target cells achieves a higher gene-editing efficiency than electroporation of the corresponding plasmid-based CRISPR-Cas9 or Cas9 mRNA/sgRNA. For instance, electroporation of RNPs achieved editing efficiencies of 87% and 94% in induced pluripotent stem cells and Jurkat T cells, respectively. By contrast, electroporation of the plasmid-based CRISPR-Cas9 system and the Cas9 mRNA/sgRNA produced a lower efficiency in induced pluripotent stem cells (20% and 32%, respectively) and Jurkat T cells (63% and 42%, respectively) [49].

4.2. Microinjection

Microinjection is the direct injection of foreign molecules into living cells using a glass micropipette at a microscopic level. As a simple mechanical procedure, microinjection has become a common laboratory technique to deliver exogenous protein or DNA into single cells. Microinjection was used to directly inject the CRISPR-Cas9 system into embryonic cells or other cells with a high reproducibility and specificity [27,50,51]. Injection of plasmids encoding Cas9 and sgRNA into the pronucleus or nucleus is the most direct method to edit genes. For example, Mashiko et al. demonstrated that microinjection of a plasmid encoding Cas9 and sgRNA into the pronucleus of mouse zygotes is a simple and convenient method to obtain a knockout mouse model within a month [52,53]. Similarly, microinjection of CRISPR-Cas9 was used to edit genes in the cells of rabbits [54], zebrafish [55], Ciona intestinalis [56], worms [57] and Aedes aegypti [58].

Because of its simplicity and accuracy, microinjection has been used to evaluate the gene-editing efficacy of different plasmid-based CRISPR/Cas9 systems targeting the same gene [59]. However, injection of circular plasmids may cause unwanted side effects when the plasmid integrates into the host chromosomes. This could be avoided by microinjection of Cas9 mRNA and sgRNA. For instance, Horig and colleagues showed that microinjection of Cas9 mRNA/sgRNA into the pronucleus has a higher efficiency than the injection of the corresponding plasmid encoding Cas9 and sgRNA or the injection of the Cas9 mRNA/sgRNA into the cytoplasm [60]. Although these results demonstrated that microinjection is an efficient physical method to

### Table 1

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<tr>
<th>Types of CRISPR-Cas9</th>
<th>Delivery strategies</th>
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<tr>
<td>Plasmid-based CRISPR-Cas9</td>
<td>Electroporation, hydrodynamic injection, microinjection, mechanical cell deformation, lipid nanoparticles, AAV, lentivirus</td>
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<tr>
<td>Cas9 mRNA and sgRNA</td>
<td>Electroporation, microinjection, lipid nanoparticles</td>
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<td>Cas9 protein and sgRNA</td>
<td>Electroporation, iTOP, lipid nanoparticles, polymer nanoparticles, CPP delivery, DNA nanostructure, gold nanoparticles</td>
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deliver plasmid-based CRISPR-Cas9 or Cas9 mRNA/sgRNA, there are several disadvantages of microinjection. First, microinjection induces cell damage and therefore requires a high level of sophistication and manual skills. Second, only a single cell can be targeted in each injection, and this method is only suitable for a limited number of cells.

4.3. Induced transduction by osmocytosis and propanebetaine (iTOP)

iTOP is a novel method to deliver native proteins and other compounds into cells. A hyperosmolar buffer containing sodium chloride and propanebetaine (a transduction compound) stimulates macro-pinocytosis, leading to cellular uptake of cargos. iTOP transduction is effective for intracellular delivery of the Cas9 protein and sgRNAs separately, or direct delivery of RNPs. RNPs have been successfully delivered into various types of primary cells using iTOP. Co-administration of Cas9 protein and sgRNA into human embryonic stem cells yields up to 26% gene-editing efficacy after two administrations [36]. Compared to other methods, including electroporation, cationic lipids and CPPs, iTOP yields lower gene-editing efficiency in primary cells [28,33,36,61,62]. In addition, the Cas9 protein is only soluble in high salt concentrations used in iTOP, and thus it is not suitable for in vivo applications.

4.4. Mechanical cell deformation

Transient membrane disruption produced by mechanical deformation promotes cellular uptake of cargos through passive diffusion. Researchers have used this method to deliver various cargoes into cells. The advantage of mechanical cell deformation is its high delivery efficiency with a low cell death rate [63,64]. Han and colleagues used a microfluidic device and optimized physical constriction to successfully deliver a plasmid-based CRISPR-Cas9 system to different cell lines. Using the microfluidic device, delivery of the plasmid-based CRISPR-Cas9 system achieved greater than 90% and 70% knockout efficiency in MDA-MB-231 (breast cancer) and SU-DHL-1 (lymphoma) cells, respectively [65]. As lymphoma cells are very difficult to be transfected, the fact that mechanical cell deformation achieves a 70% knockout efficiency in lymphoma cells is very encouraging. However, the use of this microfluidic device has not been adapted to in vivo applications.

4.5. Hydrodynamic injection

Hydrodynamic injection is the rapid injection of a nucleic acid solution into rodents via the tail vein in volumes equivalent to 8–10% of the body weight [66]. Since its discovery, it has become the simplest...
and most efficient method to deliver nucleic acids to the liver [67]. Hydrodynamic pressure is produced by rapidly administration of a large volume of a nucleic acid solution to induce the formation of temporary pores on the cell membrane of endothelial cells and facilitate the nucleic acid to enter cells [68,69]. Hydrodynamic injection has been widely utilized in a variety of applications for the delivery of proteins, small interfering RNA (siRNA), DNA, and even cancer cells. Recently, the plasmid-based CRISPR-Cas9 system was successfully delivered using hydrodynamic injection and generated efficient genetic corrections or mutations [70-72]. In one study, a plasmid-based CRISPR-Cas9 system was delivered into hepatocytes via hydrodynamic injection to correct the Fah mutation in a mouse model of the metabolic disease hereditary tyrosinemia. Briefly, a Fah-targeting sequence is cloned into the pX330 backbone, which contains cassettes for the Cas9 nuclease and sgRNA. The plasmid along with the corrected Fah DNA template was hydrodynamically injected into mice, resulting in Fah protein expression in ~1/250 liver cells [72]. Shortly after this study, the same group delivered a pX330 system co-expressing a sgRNA sequence targeting PTEN into mouse using hydrodynamic injection and showed that approximately 2.6% of sequences in the liver genome were mutated [71]. Because of the ease of hydrodynamic injection, liver cancer models and other disease models are quickly developed through hydrodynamic injection of plasmid-based CRISPR-Cas9 systems.

Despite its success in small animals, hydrodynamic injection is not a good choice for large animals. Hydrodynamic injection increases blood pressure and induces temporary cardiac dysfunction, liver expansion, and even animal death [69]. In addition to the unclear effects of hydrodynamic injection on large animals, hydrodynamic injection is difficult to be implemented in the clinic. For example, hydrodynamic gene therapy was used in a clinical trial to treat patients with cirrhosis, but the result was disappointed due to hepatotoxicity [73].

4.6. Lipid nanoparticles

Lipid nanoparticles represent one of the most commonly used delivery systems for nucleic acids, and some of them have entered clinical trials for RNAi therapy [74,75]. Typically, negatively charged nucleic acids are complexed with positively charged lipids to form lipid nanoparticles, which protect nucleic acids from nucleasce and enter target cells via endocytosis or macro-pinocytosis. Lipid nanoparticles have been explored to deliver the CRISPR-Cas9 systems to numerous cells [7,47] for therapeutic purpose or to establish knockout animal models [76,77].

For the delivery of plasmid-based CRISPR-Cas9 or the mixture of Cas9 mRNA and sgRNA, the same lipid nanoparticles that developed for plasmid and siRNA can be directly adopted without major modifications. For example, commercially available transfection lipids, such as Lipofectamine 2000, Lipofectamine 3000, RNAiMAX, can be used to deliver plasmid-based CRISPR-Cas9, Cas9 mRNA/sgRNA mixture, and even RNPs to various cell lines, including HEK293FT, U2OS, mouse ESCs, N2A, and A549, to edit target genes. Compared to Lipofectamine 2000, RNAiMAX showed better capability to deliver RNPs to cells with low toxicity [49].

Although commercially available lipids can be directly used to deliver RNPs to perform gene editing in vitro, it is generally believed that lipid nanoparticles should be modified for Cas9-sgRNA RNPs because Cas9 protein is positively charged, which comprises the complexation of cationic lipids and RNPs. In addition, RNPs, in general, show higher gene editing efficiency and less off-target effects than plasmid-based CRISPR-Cas9. Thus, considerable efforts have been devoted to develop new lipid nanoparticles for RNPs.

Zuris et al. fused a negatively charged protein, (~ 30) GFP, to Cas9 protein and demonstrated that common cationic lipids can be used to efficiently deliver the RNPs made from the modified Cas9 protein and sgRNA. Delivery of the RNPs is highly efficient and induced up to 80%
genome editing in cultured human cells after one treatment. Moreover, the modified Cas9/sgRNA RNP can be delivered into the mouse inner ear \textit{in vivo} and edited 20% of genomes in the hair cells [28]. Using the similar strategy, several bio-reducible lipids were designed to form nanoparticles with the modified RNPs for the delivery into mouse brain. These bio-reducible lipids promote endosomal release and degradation of the nanoparticles in the cytosol to release the encapsulated RNPs. Gene-editing efficiency of the lipid nanoparticles is more than 70% in cultured human HEK cells. Furthermore, the authors demonstrated that the lipid nanoparticles can efficiently deliver RNPs to mouse brain for gene-editing \textit{in vivo} [78].

4.7. Polymer nanoparticles

Polymer nanoparticles have been extensively used to deliver various types of nucleic acids including plasmid DNA, RNA, and oligonucleotides. In a recent study, a cationic polymer bPEI was covalently conjugated to Cas9 protein, which was then complexed with sgRNA to form a CRISPR nanoparticle. The polymer–conjugated Cas9 maintains its nuclease activity to induce DSB in its target gene. The polymer-based nanoparticle successfully delivered the CRISPR system to methicillin-resistant \textit{Staphylococcus aureus} (MRSA) and efficiently edited the genome. This system also demonstrated higher editing efficacy compared to unmodified Cas9/sgRNA complexed with conventional lipids [79].

4.8. Cell-penetrating peptide (CPP)

CPP is a short peptide that can translocate across cell membrane. CPP can be attached to various cargos, including CRISPR-Cas9, and deliver them into a wide variety of cells. For example, Cas9 protein can be covalently conjugated to a non-arginine based CPP, and the sgRNA complexes with another similar CPP via electrostatic interactions (Fig. 5D). The mixture of the Cas9/CPP conjugate and sgRNA/CPP complex were concomitantly delivered into the same cells and edited the genes at rates ranging from 2.3% to 16% in different cell lines, including HeLa, HEK293T, dermal fibroblasts, embryonic cells, and embryonic stem cells. Scientists also studied the mixture of the Cas9/CPP conjugate and sgRNA but did not observe gene-editing effects. This could be because that sgRNA neutralizes the positive charge of the CPP and subsequently attenuates its cell penetration capability. This result suggests that both Cas9 protein and sgRNA should be conjugated or complexed with CPP to achieve gene-editing effect [62,63].

4.9. DNA nanostructure

DNA has been utilized to construct DNA nanostructures for a variety of biomedical applications, such as imaging and targeted drug delivery. Traditionally, DNA nanostructures are assembled through Watson-Crick base-pairing of small DNA fragments. However, this assembly procedure is complicated and needs a large amount of DNA. Recently, rolling circle replication (RCR) has been developed to assemble DNA nanostructure with densely packed DNA [80]. Using the same technology, a yarn-like DNA nanoclew was recently developed for the delivery of RNPs [81]. DNA nanoclews are nanoparticles made of a DNA cage which is synthesized by rolling circle amplification. Polyethyleneimine was added to this complex to provide a positive charge to improve cellular uptake and escape from the endosome. The use of this DNA nanoclews-mediated RNP delivery achieved a mutation frequency of approximately 28% in U2OS.EGFP cells. Moreover, this delivery system disrupted approximately 25% of EGFP \textit{in vivo} following an intra-tumor injection.

4.10. Gold nanoparticles

Gold nanoparticles are a novel delivery method for RNPs. Gold nanoparticles are co-assembled with the Cas9 protein engineered with a glutamate peptide tag and sgRNA into nanoparticles. This nanoparticle-mediated delivery system achieves greater than 90% delivery efficiency and 30% gene editing efficiency in a wide variety of cell types [82]. The nanoparticle-mediated delivery of the engineered Cas9 protein and sgRNA is achieved through a cholesterol-dependent membrane fusion process that is distinct from cellular endocytosis, which may underline the remarkable delivery efficiency of this system [82]. This method provides a novel platform for transient gene editing \textit{in vitro}. However, researchers do not yet know whether this system will work in human primary cells, such as lymphoma cells.

5. Viral delivery systems for CRISPR-Cas9

As discussed above, a number of physical and non-viral delivery approaches, such as electroporation, microinjection and lipid nanoparticles have been successfully utilized for the delivery of CRISPR-Cas9 systems. Physical delivery of CRISPR-Cas9 is feasible for generating knock out cell lines and animal models, it however cannot be used for \textit{in vivo} therapeutic applications. On the other hand, regardless of the simplicity and safety of non-viral vectors, their relative poor delivery efficacy limits their \textit{in vivo} applications. By contrast, viral vectors have long been developed over three decades to deliver various nucleic acid-based therapeutics, and some of them have been approved for clinical uses. Despite their safety concerns and the possibility of introducing undesired mutations, viral delivery systems have so far the most efficient systems to deliver plasmid-based nucleic acids to mammalian cells \textit{in vivo} and \textit{in vivo} [83–86]. As a result, viral vectors have been widely applied to deliver plasmid-based CRISPR-Cas9 to mammalian cells.

5.1. Adeno-associated virus (AAV)

AAV is the most widely used viral vector for gene transduction because of its broad range of serotype specificity, ability to infect dividing and non-dividing cells, non-pathogenicity, and very mild immunogenicity [87,88]. As one of the non-enveloped and small (20 nm) paroviruses, AAV contains a single-stranded DNA genome of about 4.7 kb [89]. In addition, AAV has over 200 different molecularly engineered [90] or naturally occurring variants [91]. In 2012, Europe approved the first AAV-based gene therapy drug, Glybera, for the patients with lipoprotein lipase deficiency, suggesting the great promise of AAV for gene therapy [92]. The same AAVs developed for gene therapy have been used for the plasmid-based CRISPR Cas9.

The sequence encoding the \textit{Streptococcus pyogenes} Cas9 (SpCas9) protein and sgRNA is approximately 4.2 kb. The challenge for AAV-mediated CRISPR-Cas9 delivery is the packaging limit of AAV (approximately 4.5 kb) [93]. One solution is to use truncated SpCas9 or \textit{Staphylococcus aureus} Cas9 (SaCas9), which shows a similar gene-editing efficiency as SpCas9 but with a smaller size. The drawback of using truncated Cas9 is the lower activity (~50%) [7]. Ran and colleagues packaged an ~1 kb shorter sequence encoding SaCas9 protein and gRNA into AAV to target PCSK9 in mice and achieved ~40% gene-editing efficiency, accompanied by a remarkable decrease of PCSK9 and total cholesterol levels in the serum without signs of acute toxicity [94]. In another study, scientists packaged SaCas9 and multiple sgRNAs into AAV and showed approximately 60% gene-editing efficiency [95]. Alternatively, dual AAVs can be used to separately deliver Cas9-encoding DNA and sgRNA to overcome the packaging limit of a single AAV. Swiech and colleagues used two separated AAVs to deliver SpCas9 and sgRNAs to disrupt a single gene (Mecp2) or multiple genes (Dnmt1, Dnmt3a and Dnmt3b) in the mouse brain via stereotactic injection [96]. Similarly, the dual-AAV system was adopted to deliver CRISPR-Cas9 for the therapy of metabolic liver disease in a mouse model [97]. SaCas9 was packaged into one AAV, and sgRNA and the donor template were incorporated into another AAV. Using this dual-AAV system, ~10% mutations in hepatocytes were corrected in mice fed with a high-
protein diet, indicating that the dual-AAV system is feasible for the therapy of metabolic liver disease in animal model [97]. Despite the success of this dual-AAV strategy, the injection of two AAVs into one target cell is challenging. Therefore, Zetche and colleagues designed an AAV-mediated split-Cas9 system that can spontaneously auto-assemble Cas9 proteins inside the cells in the presence of rapamycin. Eleven potential split sites in the Cas9 protein were identified to divide the Cas9 protein to C- and N-terminal fragments. Rapamycin induction reconstitutes the split fragments into a full-length Cas9 protein, which subsequently edits genes in HEK293FT cells [98].

5.2. Lentivirus

Lentivirus is another commonly used viral vector for gene therapy. In addition to its mild immunogenicity and long-term expression of transduced genes, the biggest advantage of lentivirus is its high infection efficiency, even in non-dividing cells. This advantage is crucial for gene modification in tissues such as the liver, brain, and muscle [99]. Generally, two different types of plasmids are required to generate lentivirus. Packaging plasmids encode the structural proteins and enzymes required to generate viral particles, while the other plasmid contains foreign genetic material, such as the Cas9 and/or sgRNA cassettes, for genome-editing [100].

Lentivirus-mediated gene therapy has achieved promising results in clinical studies, such as the stable and higher levels of reconstitution of hematopoietic stem cells in most recipients with Wiskott-Aldrich Syndrome [101], and the greater than 90% reconstitution efficiency observed in patients with X-linked adrenoleukodystrophy [102]. Thus, the use of lentivirus-mediated delivery of CRISPR-Cas9 systems may obtain many benefits in further applications. To date, lentivirus-mediated delivery of CRISPR-Cas9 has achieved successful results in vitro and in vivo [86,103–105].

Lentivirus-mediated delivery of CRISPR-Cas9 is a robust tool to perform function-based screening in mammalian cells and generate knockout animal models. In general, a pool of sgRNA-expressing lentiviruses is used to generate a library of knockout cells for screening under positive and negative selections [86,103,106,107]. For example, Shen and colleagues reported a CRISPR-Cas9-mediated loss-of-function screening using a lentiviral sgRNA library (~67,405). A mutant pool of cells was generated from a mouse NSCLC cell line using the pooled genome-wide sgRNA library. Enriched sgRNAs in lung metastases were sequenced to identify the genes that may accelerate metastasis and tumor growth [108]. In addition, lentivirus-mediated CRISPR-Cas9 genome-editing has been widely used to establish various animal models of cancer [77]. Heckl and colleagues used lentivirus-mediated CRISPR-Cas9 systems to edit five genes in mouse hematopoietic stem cells, generating a model of acute myeloid leukemia (AML) [109].

A similar study performed by Blasco et al. also demonstrated that lentivirus-mediated delivery of CRISPR-Cas9 can be used for the generation of cancer models by inducing chromosomal rearrangements. In this study, lentivirus-mediated delivery of the CRISPR-Cas9 system induced the cleavage of the Alk and Eml4, leading to Alk-Eml4 rearrangements and subsequently inducing tumor formation in mice [110].

Lentivirus-mediated CRISPR-Cas9 can also be used to eradicate potential viral infections. In 2014, a lentivirus-mediated CRISPR-Cas9 system was used to remove the latent Epstein-Barr virus genomes in Burkitt’s lymphoma cells from patients. The treatment inhibits the proliferation of lymphoma cells and induces apoptosis of virus-infected cells, but cytoxicity was not observed in non-infected cells [111]. In another study, a lentivirus-mediated CRISPR-Cas9 system eliminates the HIV-1 DNA from CD4+ T cells in host patients, preventing re-emergence of HIV [112]. In addition, lentivirus-mediated CRISPR-Cas9 gene therapy demonstrated promising results in the treatment of chronic hepatitis B virus (HBV). Because the persistence of viral episomal DNA leads to HBV infection, the main strategy is to cleave viral episomal DNA and inhibit HBV replication by lentivirus-mediated delivery of the corresponding CRISPR-Cas9 systems [113–115].

6. Conclusions and perspectives

In the past few years, there has been an explosion of interest in the CRISPR-Cas9 genome-editing system because it is by far the most robust genome-editing tool. It is not surprising that scientists quickly realized its therapeutic potential for human patients. In 2016, the first clinical trial using CRISPR-Cas9 was conducted, indicating the great promise of using CRISPR-Cas9 for treating diseases with genetic disorders. However, the CRISPR-Cas9 system may face the same problems associated with gene therapy and other nucleic acid-based therapeutics. Particularly, ethical issues, off-target effects, lack of safe and efficient delivery systems are the three major barriers in using CRISPR-Cas9 for therapeutic applications in humans.

The ethical issues associated with CRISPR-Cas9 are mainly from the concern that this technology can be easily used to modify human embryos. As a result, an International Summit on Human Gene Editing was held in 2015 and suggested to establish an international deliberative group to thoroughly evaluate the risks of using CRISPR-Cas9 in humans [116]. Nevertheless, the ethical challenges of CRISPR-Cas9 are similar to those of genetic engineering and gene therapy, and the current regulations for genetic engineering and gene therapy can be adopted to establish appropriate boundaries for using CRISPR-Cas9 in humans.

Off-target effects are observed in some CRISPR-Cas9-mediated gene-editing studies, especially those studies using the plasmid-based system. Off-target effects disrupt gene functions, induce genome instability, and induce epigenetic modifications. Off-target effects are induced by off-target binding of the Cas9/sgRNA complex at sites distal from the PAM region. Particularly, off-target effect could be a potential hurdle for therapeutic applications in humans because sgRNA only targets a DNA sequence of 20 bp, and potential off-target sites may be present in the large human genome. Therefore, some strategies, such as the use of a paired Cas9 nickase, rational designed sgRNA(s), and proper selection of the targeting site, should be considered to avoid potential off-target effects of the CRISPR-Cas9 system.

In addition, proper selection of a delivery system and the type of CRISPR-Cas9 can also reduce the off-target effect. In general, non-viral delivery system has lower off-target effects than that of viral delivery system [117]. For instance, a recent study showed that lipid-mediated Cas9 mRNA delivery has lower off-target effects than lentivirus-mediated Cas9 delivery [118]. Compared to the plasmid-based CRISPR-Cas9 and Cas9mRNA/sgRNA, the complex of Cas9 protein and sgRNA shows less off-target effects. For example, CPP-mediated delivery of Cas9 protein and sgRNA induced efficient gene-editing with lower off-target mutations compared to the transfection of plasmid-based CRISPR-Cas9 [62].

Similar as gene therapy, delivery of CRISPR-Cas9 systems to target tissues or cells in human body is the biggest challenge for its therapeutic applications. Development of safe and efficient delivery systems is therefore crucial for the success of CRISPR-Cas9 in clinics. Over the last few years, various delivery systems have been exploited. The delivery strategies of the CRISPR-Cas9 systems could be similar to that have been developed for nucleic acids and proteins in the past three decades. For example, delivery strategy of the plasmid-based CRISPR-Cas9 system is the same as that of plasmid-based gene therapy. Viral and non-viral vectors developed for gene therapy can be directly used for the plasmid-based CRISPR-Cas9 system without further modifications. Delivery of the Cas9 mRNA and sgRNA is challenging but similar to siRNA delivery. The delivery systems developed for siRNA could be therefore used for the delivery of Cas9 mRNA and sgRNA after minor modifications. By contrast, delivery of the Cas9 protein/sgRNA complex is very different from the existing delivery systems for nucleic acids because Cas9 protein is a positively charged protein. Various strategies have been developed to modify Cas9 protein so that it can form a stable
complex with sgRNA and non-viral vectors. Pioneer studies demonstrated that chemical modification of Cas9 protein does not attenuate its nuclease activity. Nonetheless, scientists have learned a great deal about nuclease acid delivery, and many of the experiences can be greatly helpful to develop a safe and efficient delivery system for the CRISPR-Cas9-based therapeutics.

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