Different Methods of Delivering CRISPR/Cas9 Into Cells

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Contents

Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated system (Cas) is comprised of repetitive bases followed by short fragments of DNA from a previously invading organism that provide immunity to the most prokaryotic organisms. An RNA-dependent spacer is required for CRISPR/Cas9 to recognize the target DNA. Delivery of the CRISPR/Cas9-guide RNA (gRNA) complex to any cell results in modification of the target sequence. The CRISPR/Cas9-mediated genome editing technique is currently in the spotlight and has several research interests, including molecular medicine and agriculture. There are several factors that hinder the delivery of this complex, such as the large size of the plasmid or high dosage of the chemical agent. There are several methods available to deliver CRISPR/Cas9 and its components to the target cells. It includes viral, non-viral and physical methods to deliver plasmid or ribonucleoprotein (RNP) of CRISPR components. But in vivo CRISPR/Cas9 delivery remains challenging to the researchers due to insertional mutagenesis, targeted delivery, immunogenicity, and off-targets. However, studies suggesting that the CRISPR/Cas9-RNP delivery can

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overcome these hurdles. Here, we review the various methods for delivery of CRISPR/Cas9 and gRNA to several cell lines, highlighting the limitations of each approach, and suggest possible alternative methods.

1. INTRODUCTION

The growth of genome editing technologies involving zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated system (Cas) over a relatively short period has been rapid in many fields of research. The notion of genome editing by engineering nucleases has recently become popular. Site-specific alteration of the genome has transformed science and holds great promise for molecular medicine. Generally, genome editing indicates the insertion, deletion or replacement of a gene in the living organism using engineered nucleases. For the past two decades, researchers have used an array of proteins that recognize specific DNA sequences in the genome to efficiently edit the DNA using nucleases that result in DNA double-strand breaks (DSBs).[1] This activates DNA repair pathways including homologous recombination (HR) and non-homologous end joining.[2–4] Some of the important features of engineered nucleases are given in Table 1.

1.1 Programmable Nucleases

The first class of programmable nuclease was ZFNs, which can bind to specific target DNA through zinc finger proteins (ZFPs) (Fig. 1A). This 30-amino acid structure has a type II restriction endonuclease domain called FokI that is used to cleave the DNA. Due to the large number of ZFPs, the probability of off-target binding is very high and leads to undesired mutations.[17] A few years after the discovery of ZFNs, the search for a better alternative to ZFNs led to the introduction of TALENs. A typical TALEN involves fusion of a transcription activator-like effector (TALE) and a DNA cleavage domain (FokI) (Fig. 1B).[8] This complex introduces site-specific cleavage upon delivery into the cells. Initially, the wild-type FokI restriction enzyme was used to introduce the cleavage; however, in subsequent studies, FokI domain variants with mutations have also been employed to improve the cleavage activity[18] and specificity.[19,20] After much research on ZFNs and TALENs, the CRISPR/Cas9 system was introduced in the field of genome editing technology.

Table 1 Significant Features of Engineered Nucleases

			CRISPR Variants				
Type of Nuclease	**ZFN**	**TALEN**	**Cas9**	**Cpf1**	**C2c1**	**C2c2**	**Cas13b**
DNA binding partner	ZF proteins	TALE proteins	crRNA and tracrRNA	crRNA	crRNA and tracrRNA	crRNA	crRNA
Endonuclease	FokI	FokI	Cas9	Cpf1	C2c1[a]	C2c2[a]	Cas13b[a]
PAM	NA	NA	NGG	TTTN	TTN	PFS[b]	Flanking PFS[b]
Target length (nt)	18–36	30–40	20	23	20	28	30
Binding specificity ratio	1:3	1:1	1:1	1:1	1:1	1:1	1:1
Off-target effect	High	Low	Inconsistent	Low	Low	Low	Unknown
Reference	5–7	8	9–11	12	13	14,15	16

[a]RNA-guided RNA editing nuclease.
[b]Protospacer flanking sequence.

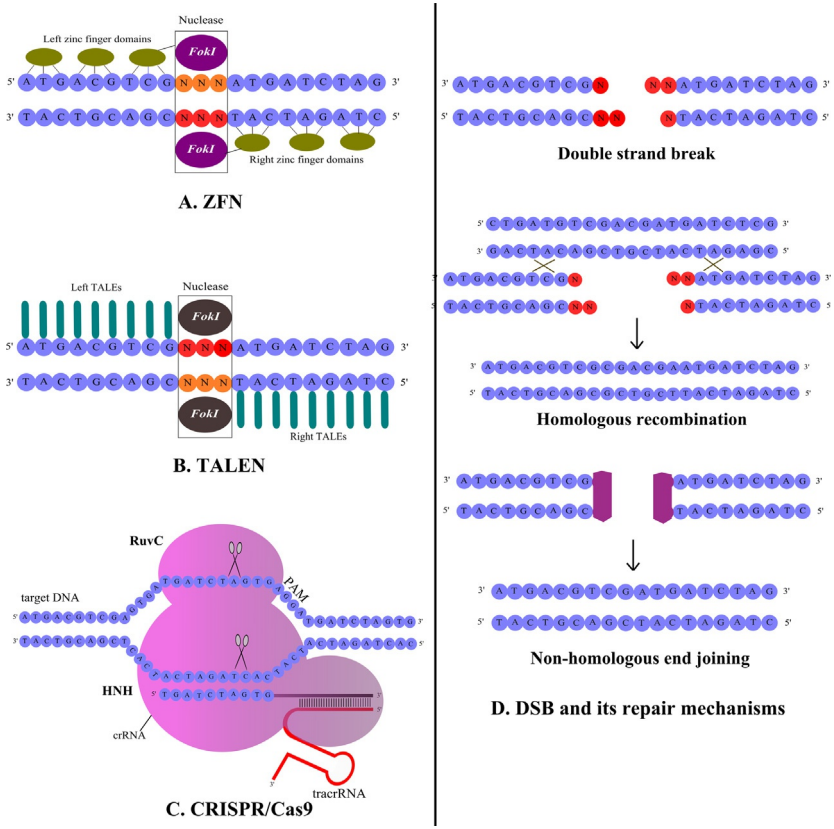

Fig. 1 Summary of genome editing tools. (A) Schematic representation of the ZFNs targeting the DNA molecule. Each individual right and left zinc finger domain targets each codon of target DNA. A FokI enzyme is attached to the both zinc fingers to mediate the site-specific cleavage. (B) Diagrammatic depiction of genome editing by TALENs. Similar to ZFNs, here the individual TALE protein recognizes a specific nucleotide of the target DNA and a FokI endonuclease is linked to TALE proteins for the cleavage. (C) Diagram showing the CRISPR/Cas9-mediated genome editing. The Cas9 nuclease has two domains, RuvC and HNH which is used to create the DSB. Here the Cas9 endonuclease is guided by crRNA and tracrRNA to cleave the target site. The crRNA and tracrRNA together called gRNA. (D) Illustration images of the DNA repair mechanisms after the site-specific cleavage by ZFNs, TALENs or CRISPR/Cas9.

Originally, the CRISPR system is an adaptive immunity of bacteria and archaea for protection against intrusive organisms.[21] Subsequently, CRISPR systems have been implemented in genome editing technology to target a specific locus. Three types and 10 subtypes of CRISPR/Cas have been suggested, and each type has core proteins and a definite module.[22] Among all available types, Cas9, which belongs to type II, is the most widely

studied for genome editing.[23–25] CRISPR/Cas9 is easy to design and manipulate and efficient for genome editing in a wide variety of organisms.[9] Cas9 requires CRISPR RNA (crRNA) and trans-activating crRNA to direct the nuclease for sequence-specific cleavage. A protospacer adjacent motif (PAM) containing 2–5 nucleotides that are essential for targeting the DNA (Fig. 1C).[26]

1.2 Genome Silencing vs Genome Editing

The genome editing technique involves the complete eradication of gene expression and generation of DNA DSBs that result in "knockout" of a particular gene. In contrast, "knockdown" of the gene of interest by RNA interference (RNAi) or gene silencing methods is the most studied method among eukaryotes. The concept of RNAi was discovered in the worm *Caenorhabditis elegans*, a nematode in which the response to dsRNA results in target-specific gene silencing.[27] This novel research led to the concept that a number of distinct homology-dependent gene silencing events might share a general biological function. The mechanism behind this approach is the production of exogenous short interfering RNAs or short hairpin RNAs that are specific to the target gene. The resultant complex is handled by Dicer, affecting the downregulation of gene expression.

Recently, catalytically dead Cas9 (dCas9) was produced that does not contain endonuclease activity. dCas9 was used for gene silencing in mammalian cells via CRISPR interference (CRISPRi) and in *E. coli*.[28] The differentiation conditions of human pluripotent stem cells (hPSCs) can be regulated by dCas9 to study its pathway.[29,30] The main advantages of CRISPRi are the ability to target multiple genes and fewer off-target effects. Both knockdown and knockout approaches are essential and based on the research application.

1.3 Advantages of CRISPR Over ZFN and TALEN

The CRISPR/Cas9 system is a powerful technique that has been utilized in stem cells, knockout strategies,[31] generation of engineered animal models,[32] and cancer modeling.[33] The implications of using the CRISPR/Cas9 technique to edit the genome are relevant to several essential strategies including the development of antiviral therapies, restoration of unfavorable mutations, and model systems. Recently, the essential characteristics of mouse and human developmental lineage-restricted *BCL11A* gene were annotated using pooled CRISPR/Cas9–gRNA. As a result, erythroid enhancer of *BCL11A* was verified as a potential therapeutic target for the reinduction of fetal hemoglobin.[34]

The prerequisite for engineering of ZFNs and TALENs is the design of proteins that target DNA molecules, followed by nuclease assembly. One nuclease is used for right ZFN/TALEN and another for left ZFN/TALEN. In contrast, the CRISPR method of editing the genome is based on a single gRNA without the requirement for enzyme engineering. The Cas9 enzyme remains the same for all targeting sequences, and only the gRNAs must be customized. The highlight of the CRISPR/Cas9 system is the short gRNA sequence of approximately 20 base pairs targeting the specific region of a genome. Furthermore, gRNAs are easier and simpler to customize than other nucleases. Because of the versatility and specificity of the CRISPR/Cas9 system, laboratories with limited resources can utilize this technique for a wide variety of applications. Also, the short length of gRNA makes it more convenient to deliver to the target site than the high-MW ZFNs and TALENs. Several questions have arisen among the scientific community over the last few years, mainly regarding off-target activity and translation to the clinic, for example: Does the enzyme cleave random site(s) other than the target? What is the strategy for the CRISPR/Cas9 system to clear clinical trials? To overcome the off-target effects, whole-genome sequencing and deep sequencing strategies should be involved in the identification of off-targets induced by genome editing. Such analyses are important for human safety and to remove obstacles to clinical trials.

2. DELIVERY METHODS OF CRISPR/Cas9 FOR GENOME EDITING

Currently, there are several methods for delivering CRISPR systems in vivo and in vitro (Table 2). Both delivery vectors and physical methods are widely implemented for the efficient delivery of CRISPR/Cas9-mediated genome editing. Delivery vectors such as viral and non-viral vectors can accommodate mRNA or plasmid expressing the nucleases to target cells or tissues. Alternatively, physical methods including electroporation, laser, ballistic delivery, physical energy, or microinjection can be exploited for the delivery of nuclease into cells.[43] Basically, non-viral vectors are preferable because of the limitations of viral vectors such as carcinogenesis,[44] limited encapsulating capacity,[45] and immunogenicity.[46] Because of the low delivery efficiency in vivo, only a limited number of non-viral vectors for gene therapy have entered into clinical trials.[47,48] In this context, we summarized methods for delivery of CRISPR/Cas9 systems into cells (Fig. 2).

Table 2 Summary of CRISPR/Cas9 Delivery Systems for Various Applications in Stem Cells

Delivery Methods	Stem Cells	Target Gene(s)	Applications	Reference
Lentiviral vector	hPSCs	*NF1*, *MED12*, *CUL3*, *TADA1*, and *TADA2B*	Gene knockout	35
Adeno-associated viral vector	hHSCs	*HBB*	Gene correction	36
Electroporation	iPSCs	*HBB*	β-Thalassemia	37
		RPGR	Retinitis pigmentosa correction	38
	hPSCs	*SH2B3*	RBC production	39
		AKT2, *CELSR2*, *CIITA*, *GLUT4*, *LINC00116*, and *SORT1*	Comparative study between TALEN and CRISPR	40
Nucleofection	iPSCs	*CFTR*	Cystic fibrosis	41
	hHSCs	*B2M* and *CCR5*	Gene knockout	42

2.1 Viral-Mediated Delivery

Viral-mediated delivery is accomplished through two mechanisms: infection and replication. During the infection stage, a virus can recognize and enter a specific cell, and the viral genome will be released into the nucleus (in case of DNA) or cytoplasm (in case of RNA) for replication. After replication of the viral genome in the cells, reproduced virions exit the cells. The infection stage starts again in neighboring cells, and the infection–replication cycle continues.[49] The virus containing the delivery material (programmed genome editing nuclease) is transported to the target cells and gene therapy can be achieved by genome editing. A number of viral vectors have been developed such as adenoviral vectors (AdVs), adeno-associated viral vectors (AAVVs), and lentiviral vectors (LVs).

2.1.1 Adeno-Associated Viral Vector-Mediated Delivery

Delivery of Cas9 into different cell lines using an AAVV system has been reported.[50–52] A suitable guide RNA (gRNA) and SaCas9

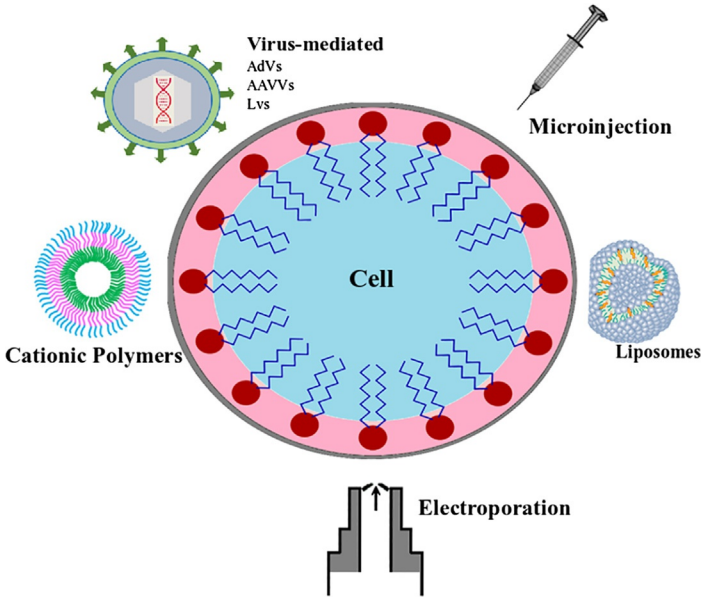

Fig. 2 Schematic representations of the different delivery methods for the successful delivery of CRISPR/Cas9 and gRNA plasmids to the cells.

(Cas9 from *Streptococcus aureus*) nuclease were successfully delivered to the livers of adult mice using the AAVV-mediated delivery system,[53] leading to successful disruption of the *Pcsk9* gene in mouse liver (40% disruption). Furthermore, SpCas9 (Cas9 from *Streptococcus pyogenes*) was also delivered and showed successful mutation of the *Pcsk9* gene with a rate higher than 50%.[54] Knockout of the *Pcsk9* gene led to a low cholesterol level in the mice, as PCSK9 protein is involved in LDL receptor degradation. In another study, delivery of CRISPR/Cas9 through AAVVs was achieved with restoration of functional deficiency of mouse muscle.[55] The mRNA or cDNA of SaCas9 was encapsulated in either AAVV8 or AAVV9 for delivery into tibialis anterior muscle of *mdx* mice for correction of Duchenne muscular dystrophy (DMD).[55–57] The mouse model *mdx* has a mutation in exon 23 which is responsible for DMD. An AAVV-mediated delivery method was proposed to treat DMD in the mouse model by employing CRISPR/Cas9-based editing technique. SaCas9 was used for in vivo genome editing. On the other hand, AAVV expressing sgRNAs were also prepared to target introns 22 and 23 to create deletion. These introns flanking to the exon 23 on both the sides and thus the deletion of the two introns resulted in the complete elimination of exon 23. Equal volume of Cas9 and sgRNA were injected into

the muscle of *mdx* mice and harvested after 8 weeks for analysis. End-point PCR and droplet digital PCR showed the expected deleted region (approx. 1171 bp) and approximately 2% of all alleles showed exon 23 deletion. Sanger and deep sequencing results further confirmed the deletion of exon 23. The results showed that muscle force was enriched and muscle biochemistry was strongly improved. This research demonstrated the potential of CRISPR/Cas9 delivery by AAVV as a prospective therapy to treat DMD.

The AAVV delivery system is commonly used for gene therapy due to its long-lasting transgene expression, mild immune response, high infection efficiency, and general safety and is now being tested in clinical trials.[58] However, the limited packaging range of approximately 4.7 kb makes it inappropriate for larger Cas9 variants.[26,59] To overcome this limitation, several distinct solutions have been proposed. Smaller orthologs of Cas9 (SaCas9) with a size of 3.3 kb have been bundled into a single AAVV,[53] and another ortholog called *Streptococcus thermophilus* Cas9 (St1Cas9), which was also 3.3 kb, was similarly packed into a single AAVV.[60,61] Furthermore, other studies employed the dual-AAVV system in which one AAVV delivers SpCas9 and another delivers gRNA.[62,63] The disadvantage of this system is the reduced probability of delivering both viral vectors to the same cell, which decreases the efficiency of delivery.

2.1.2 Lentiviral Vector-Mediated Delivery

The simultaneous delivery of both Cas9 and gRNA into the host cells can be accomplished by lentiviral vectors. Here, a cell line expressing Cas9 is not a prerequisite for the co-delivery of Cas9 and gRNA.[35] Basically, LVs are initially acclimatized from HIV-1, which yields an extremely effective viral vector due to its tropism, capability to infect post-mitotic cells, and larger cargo capacity of 9.7 kb.[64] Ex vivo gene therapy can also be achieved using LVs, particularly in T cells (T lymphocytes) and hematopoietic stem cells (HSCs). In a recent study, LVs were exploited to treat β-thalassemia and Wiskott–Aldrich syndrome and for T-lymphocyte immunotherapy.[65] Use of integrase-deficient lentivirus vectors that have viral LTRs and integrase mutations significantly decreases the risk of insertional mutagenesis, but does not entirely eradicate the risk.[66–68] Currently, various research groups are utilizing LV-mediated delivery of gRNA and Cas9 to create knockout libraries.[35,69] These LVs can incorporate into the target genome without changing its original form and express both gRNA and Cas9.

LVs have great potential for further applications in in vivo gene therapy. Proof of concept of in vivo therapy using an LV-dependent system for the

delivery of CRISPR–Cas9 system is now being developed. Recently, a pre-packed LV-Cas9 protein system have been developed to edit CCR5 gene in TZM-bl cells.[70] Also, they designed a specific sgRNA targeting the long terminal repeat of HIV to disrupt HIV proviral DNA in the J-LAT cells. The CCR5 sgRNA displayed minimal off-target on chromosome 4. The above evidence has further confirmed the efficiency of LV-mediated delivery of Cas9 for safe access for gene therapy applications. In another study, dual LV system was developed to get effective gene knockout in difficult to transfect human and mouse cell lines and primary cell lines.[71] This dual vector consists of an inducible sgRNA cassette and a constitutive expression Cas9 vector linked with mCherry. With the help of this system, they induced mutation in Trp53 in primary HSPCs and resulted in the growth of lymphoma that is driven by MYC. Thus, the combined action of dual LV and CRISPR/Cas9 systems can be utilized for annotating novel mutations in tumor suppressor genes and oncogenes that can accelerate tumor development.[71] However, further analysis might require that the delivery of the substrates and effector proteins is well balanced and timed. Nuclease-deficient Cas9 (dCas9) delivery can be employed, or non-integrating LVs can be used for nuclease applications. Also, problems concerning the integration of Cas9 into the target genome using LVs are not completely resolved.[72]

2.1.3 Adenovirus-Mediated Delivery

Adenoviridae is a family of adenoviruses that were isolated from human adenoid tissues in 1953. Generally, the concept of AdV-mediated delivery can be used for in vivo and in vitro applications.[73] Delivery of RNA-guided nuclease into several cell lines was successfully accomplished.[74] The researchers investigated second-generation fiber-modified AdVs expressing gRNA or the Cas9 component transduced into a recombinant allele or the safe harbor gene called *AAVS1* that can be generated to high titers.[74] Although AdVs generate immune responses when transduced into hepatocytes, genome editing of somatic cells by AdVs can generate prolonged liver phenotypes.[75] The SpCas9 system was delivered by AdV to target the *Pten* gene of mouse liver, and the genome editing efficiencies of AdVs and hydrodynamic injection-mediated delivery were compared. After 2 weeks of treatment, the hydrodynamic injection-mediated delivery was less efficient than AdVs. In addition, steatohepatitis and hepatomegaly were observed after 4 weeks of AdV genome editing treatment.[75]

2.2 Non-viral Vectors

Several non-viral vector systems have been developed and successfully employed for safe delivery of CRISPR/Cas9 to cells. Noteworthy delivery methods include polymeric materials, liposomes, cell-penetrating peptides (CPPs), and cationic nanocarriers. The main advantages of non-viral vectors are the capability to accommodate components of a large size for delivery, their reduced or non-hazardous nature, and easy generation. For these reasons, many scientists select this type of system for the delivery of nucleases.

2.2.1 Cationic Vectors

There are two broad categories of cationic based vectors: cationic polymer-based and cationic lipid-based vectors. The probability of uptake of the delivery components (Cas9 or gRNA plasmid) is high because of their anionic nature. Thus, by nature, the cationic vectors are more suitable and efficient for delivery of anionic gRNA or Cas9 to the cells. Effective delivery of Cas9 was achieved using cationic polymer nanoparticles (NPs).[76] Polyethyleneimine (PEI) is a common cationic polymer that is used for the delivery of CRISPR components. The molecular weight (MW), the amount of branched or linear structure, and structural characteristics are significant properties that determine the transfection efficiency and toxicity to the cells. Also, the secondary amine present in the PEI helps to retain the DNA in the cells.[76] The stability of PEI is based on its high MW, high number of branched structures, and high cationic charge; however, it could cause toxicity to cells. PEI-based delivery of Cas9 for in vivo targeting of tumor suppressor genes (*Pten*, *Trp53*, *Nf1*) in mouse brain successfully caused gene disruption.[77]

Cationic lipid-based vectors are also useful for the efficient delivery of Cas9 and gRNA. For instance, gRNA and Cas9 ribonucleoprotein (RNP) complexes were successfully delivered to mouse hair cells by cationic liposomes, and 80% gene modification was achieved.[78] The vectors were injected into the inner ear rather than using the intravenous route, as injection is efficient and similar to the clinical technique. Another technique for the delivery of CRISPR/Cas9 is by employing 7C1 NPs, which can be produced by mixing low-MW PEI with C15 epoxide-terminated lipids. This conglomeration method enhanced the mutation efficiency in the cardiovascular and pulmonary endothelium and generated Cre-based Cas9 mice.[51]

2.2.2 Cell-Penetrating Peptides

Technical limitations such as susceptibility to enzymatic degradation, anionic nature, and high MW make DNA delivery into target cells very difficult. CPPs have several advantages and are proven to be successful in delivering therapeutics against multiple diseases.[79] CPPs carrying Cas9–gRNA have been used for successful genome alteration in various cell types (HeLa cells, dermal fibroblasts, human embryonic stem cells (hESCs), HEK293T cells, and embryonic carcinoma cells) with low off-target activity. In one study, CPP was conjugated with gRNAs and recombinant Cas9 proteins to cause gene disruptions at the target site in human cells lines. This approach has some advantages over other non-viral methods because of its chemical reagent-free delivery and fewer off-target effects.[80]

2.2.3 Other Non-viral Methods

Several studies have involved CRISPR/Cas9 delivery using cationic NPs. Recently, CRISPR/Cas9 was delivered by bioreducible lipid NPs with 70% genome efficiency.[81] A hydrodynamic-based injection for the delivery of gRNA–Cas9 complex to target cells was recently reported.[82] Hydrodynamic injections are high-volume injections that are delivered at high speed into the vasculature for efficient delivery of Cas9 and gRNA plasmids and have been used in many in vivo analyses targeting the liver.[82,83] In this study, a cancer model had been generated using CRISPR/Cas9 in mice. A plasmid co-expressing Cas9 and sgRNA was injected to the liver by the hydrodynamic injection. The plasmids target the tumor suppressor genes *p53* and *Pten*. The DNA sequencing results confirmed the biallelic mutations happened in both *p53* and *Pten*.[83] This data exhibits the possibility of direct mutation of tumor suppressor genes in the liver using CRISPR/Cas9 that explains the progress in liver cancer models and genomics. However, this method is not extensively used because of possible damage to the heart and liver.[84]

2.3 Physical Methods

There are several physical methods for delivery of Cas9 nuclease into cells including microinjection and electroporation. Because of its target specificity, high reproducibility, and simplicity, this approach is a promising technology for gene therapy without the limitations associated with viral vectors. Also, these methods can be readily applied to different cell lines in vivo and in vitro.[85] For direct delivery of the constructs to a target cell, a needle with diameter ranging from 0.5 to 20 μM is generally inserted into

the zebrafish[86] and mosquito embryos.[87] The overall experiment is aided by a special microscope equipped with a micromanipulator.[86–88] Alternatively, electroporation generates pores on the cells and thus enables the plasmids to enter the cells. Usually, the electroporator is used to produce electric pulses to create pores.[85,89–91] Efficient knockout of the *SH2B3* gene in hESCs was accomplished by co-transfection of Cas9 and gRNA plasmids. In this knockout analysis, 25 μg of each plasmid was electroporated into hESCs. The resultant suppression of the *SH2B3* gene in hESCs enhanced erythroid expansion and differentiation. Similarly, *SH2B3* gene suppression in both HSCs and pluripotent stem cells (PSCs) resulted in erythroid expansion.[39] CRISPR/Cas9-based targeting of the cystic fibrosis (CF) gene (*CTFR*) in patient-derived induced pluripotent stem cells (iPSCs) significantly corrected the phenotype. The CRISPR/Cas9 plasmid, gRNA for *CTFR*, GFP, and donor template were co-nucleofected into iPSCs from CF patients. Consequently, the corrected iPSCs differentiated into epithelial cells, and their function was analyzed.[41]

3. OPPORTUNITIES AND CHALLENGES IN CRISPR/Cas9 DELIVERY TO STEM CELLS

The improvement of target-specific nucleases as research tools parallels recent developments in iPSCs. Reconsidering the Cas9 technique as an engineered nuclease eliminates the barriers that restricted the prospects of genome editing by offering a common platform. The main advantage of this system over ZFNs and TALENs is that the DNA-targeting specificity is determined by the gRNA and does not involve the arduous process of designing DNA binding proteins. Thus, integrating the cellular adaptability of iPSC differentiation with straightforward genome editing by the CRISPR/Cas9 system is a powerful technique and has become a benchmark tool in stem cell research and disease modeling.[92,93]

An inducible cassette expressing Cas9 with gRNA was delivered to the *AAVS1* locus to enable the generation of isogenic hPSCs. Doxycycline was used to induce the expression of Cas9.[94] Here, iCRISPR (combination of TALEN and CRISPR/Cas9) was developed for the efficient biallelic knockout of hPSCs and for knock-in specific alternations in the nucleotide to generate disease models in hPSCs. First, AAVS1-TALEN along with two donor constructs were electroporated to the first intron of the AAVS1 in hESCs and hiPSCs cell lines. A high biallelic mutation was observed in >50% of clones which was confirmed by Southern blot and qRT-PCR

revealed the Cas9 expression after doxycycline induction to all iCas9 clones. Furthermore, the pluripotency markers (NANOG, OCT2, and SOX2) were expressed in iCas9 hPSCs and retained its stemness property. Altogether, the iCRISPR can be used to study the pleiotropy in human disease and to examine the complex genetic interactions. Also, it has the ability to assist the high-throughput analysis in hPSCs. A loss-of-function study of *EZH2* was performed, and haploinsufficiency for *EZH2* was revealed in hematopoietic differentiation.[95] Recently, effective delivery of CRISPR/Cas9 into human hematopoietic stem and progenitor cells (HSPCs) was accomplished by electroporation with chemically modified gRNA targeting the cells.[96] The chemical modifications include 2′-*O*-methyl, 2′-*O*-methyl 3′ phosphorothioate, or 2′-*O*-methyl 3′ thioPACE were integrated at the 5′ and 3′ ends.[96] Another recently published study described targeting the *HBB* gene in HSPCs.[36] HR-dependent genome editing in $CD34^+$ HSPCs from peripheral blood was performed using CRISPR/Cas9 ribonucleoprotein paired with recombinant AAVV serotype 6 (rAAVV6) donor. To attain efficient gene editing, the chemically modified sgRNAs were co-transfected with Cas9 RNP or mRNA. The *HBB*-single-stranded AAVV6 (ssAAVV6) was tagged with GFP to check the transfection efficiency. Both RNP and mRNA resulted in high INDEL when HSPCs was electroporated. After the electroporation of Cas9 RNP, *HBB*-ssAAVV6 donor was introduced and 29% of stable GFP expression was obtained. On the other hand, mRNA system showed lower efficiency (15%). Also, the off-target and cytotoxicity were low for RNP platform. The above data revealed that CRISPR/Cas9-based editing of the *HBB* gene in HSCs has the potential for translation to clinical medicine.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

Both non-viral and viral vectors have been implemented for the delivery of CRISPR/Cas9 for various applications. Non-viral vectors for the delivery of CRISPR/Cas9 in the form of plasmid, mRNA or protein along with gRNA has appeared as a potential delivery approach with intrinsic strength and ability. On the other hand, Cas9 delivery approaches by viral vectors have attained some level of in vivo therapeutic gene editing but effective editing remains a challenge. The effective delivery of CRISPR components to the target is the major challenge with respect to specificity and efficacy. Depending on the nuclease expression and modes of delivery, both immune response and off-targets are possible. Both non-viral and viral

methods are being assessed for the delivery of CRISPR/Cas9 to cells in vivo or ex vivo. On the basis of the evidences, the in vivo delivery of Cas9 via PEL, CPP, AAV, and cationic liposomes are successful. Furthermore, hydrodynamic injection has been applied to carry a large size of CRISPR/Cas9 plasmid into mouse hepatic tissue.[82,83] On the other hand, physical methods include microinjection and electroporation have succeeded in the uptake of plasmids to the target. Additionally, a formulation consist of cationic surfactant benzalkonium chloride and non-ionic poloxamer CRL 1005 has gone into clinical trial phase II/III.[97]

The non-viral delivery of Cas9 plasmid or protein decreases off-targets and immune response in vivo. Also, these vectors have lesser advantage over viral vectors in various conditions specifically gene knock-in. However, constant expression of CRISPR/Cas9 using viral vectors induces off-targets, immune response and thus must be improved. In the near future, donor template and sgRNA may be prepared into viral vectors for constant expression and transient CRISPR/Cas9 can be introduced via non-viral and administered for efficient DNA cleavage. Thus, the blending of multi-administered non-viral vectors with viral vector will be the most favorable method for molecular medicine based on CRISPR/Cas9 technology.

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