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Non-viral and viral delivery systems for CRISPR-Cas9 technology in the biomedical field

Zhi-Yao He[1], Ke Men[2], Zhou Qin[1], Yang Yang[2], Ting Xu[1*] & Yu-Quan Wei[2]

[1]*Department of Pharmacy, and Cancer Center, West China Hospital, Sichuan University and Collaborative Innovation Center of Biotherapy, Chengdu 610041, China;*

[2]*State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University and Collaborative Innovation Center of Biotherapy, Chengdu 610041, China*

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The clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR-Cas9) system provides a novel genome editing technology that can precisely target a genomic site to disrupt or repair a specific gene. Some CRISPR-Cas9 systems from different bacteria or artificial variants have been discovered or constructed by biologists, and Cas9 nucleases and single guide RNAs (sgRNA) are the major components of the CRISPR-Cas9 system. These Cas9 systems have been extensively applied for identifying therapeutic targets, identifying gene functions, generating animal models, and developing gene therapies. Moreover, CRISPR-Cas9 systems have been used to partially or completely alleviate disease symptoms by mutating or correcting related genes. However, the efficient transfer of CRISPR-Cas9 system into cells and target organs remains a challenge that affects the robust and precise genome editing activity. The current review focuses on delivery systems for Cas9 mRNA, Cas9 protein, or vectors encoding the Cas9 gene and corresponding sgRNA. Non-viral delivery of Cas9 appears to help Cas9 maintain its on-target effect and reduce off-target effects, and viral vectors for sgRNA and donor template can improve the efficacy of genome editing and homology-directed repair. Safe, efficient, and producible delivery systems will promote the application of CRISPR-Cas9 technology in human gene therapy.

genome editing, CRISPR, Cas9, viral vector, non-viral vector, gene therapy

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INTRODUCTION

Targeted genome editing technology can be used to edit a specific genomic locus for genetic knock-out or correction (Chen et al., 2017; Joung and Sander, 2013; Urnov et al., 2010). The target of genome editing therapeutics is genomic DNA rather than a kinase (protein) of a targeted kinase inhibitor or antigen of an antibody (He et al., 2016; Li et al., 2016; Osakabe et al., 2016; Topalian et al., 2012). Consequently, therapeutics based on genome editing technology directly target the root cause of many diseases, rather than secondary effects (Figure 1) (Gaj et al., 2013; Hille and Charpentier, 2016; Savić and Schwank, 2016). Moreover, some previously undruggable targets can now be treated by targeted genome editing technology, making this system important in the targeted therapy field (Cox et al., 2015; Savić and Schwank, 2016). The clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR-Cas9) system was discovered as part of the immune response by bacteria; some modified CRISPR-Cas9 systems have been shown to be ro-

*Corresponding author (email: tingx2009@163.com)

bust and precise in mammalian cells (Cheong et al., 2016; Cox et al., 2015). Therefore, CRISPR-Cas9 systems may be useful for treating human diseases, including hereditary diseases, cardiovascular diseases, metabolic diseases, degenerative diseases, cancer, and infectious diseases, among others (Cox et al., 2015; Croce et al., 2016; Jiang et al., 2017; Munshi, 2016; Yang et al., 2016; Zhang and Wang, 2016). Recently, institutions in China (West China Hospital) and the US are planning to perform clinical trials for cancer therapy using CRISPR-Cas9 technology (Cyranoski, 2016; Deng et al., 2016; Reardon, 2016). Numerous commercial and synthesized reagents can transfer CRISPR-Cas9 system into cells for efficient targeted genome editing *in vitro*, but scientists must develop approaches for delivering the CRISPR-Cas9 system into target organs of animals or humans *in vivo* (Cox et al., 2015). Furthermore, on-target and off-target effects are related to the delivery vectors of the CRISPR-Cas9 system (Yin et al., 2016; Zhang and Li, 2016). Therefore, delivery systems are crucial for eventual commercialization (drugs) based on CRISPR-Cas9 technology. This review focuses on delivery systems used to mediate CRISPR-Cas9 constructs into cells *in vitro* or animals *in vivo*.

DIRECT TRANSPORT OF CRISPR-CAS9 SYSTEM

Co-microinjection with Cas9 and sgRNAs

By co-microinjection of *Streptococcus pyogenes* Cas9 (*Sp*Cas9) mRNA and sgRNAs targeting *Ppar-γ* into one-cell-stage embryos, Niu et al. successfully achieved precise gene targeting in cynomolgus monkeys. Furthermore, 6 of 15 embryos harbored simultaneous disruption of two target genes (*Ppar-γ* and *Rag1*) in one step via this co-microinjection, as the CRISPR-Cas9 system functioned well in monkey embryos (Niu et al., 2014). A platform based on embryo co-microinjection of nCas9n protein/mRNA, sgRNAs, and/or a single-stranded DNA (ssDNA) template enabled the generation of knock-out alleles via non-homologous end-joining (NHEJ) and knock-in alleles via homology-directed repair (HDR) in African turquoise killifish. This efficient genome engineering approach for the short-lived killifish provided powerful genetic tools for studying vertebrate aging and aging-related diseases (Harel et al., 2016; Jao et al., 2013). Zhang et al. designed two target sites in conserved regions of the vitamin D receptor (VDR) gene, and then co-microinjected *Sp*Cas9 mRNA and sgRNAs targeting *VDRT1* and *VDRT2* into one-cell-stage embryos of C57BL/6 mice. Twelve mice showed VDR-targeted disruption and 8 mice were biallelic knock-out as validated by a T7E1 assay and DNA sequencing analysis (Zhang et al., 2016). These results indicate that CRISPR/Cas9-mediated knock-out can be achieved by co-microinjection of sgRNAs and Cas9 mRNA into one-cell embryos. However, *in vitro* oocyte injection may improve the efficiency of gene editing in zebrafish and increase the rate of generating heritable mutants in zebrafish compared with one-cell embryo injection, particularly for sgRNAs with low targeting efficiency. *Sp*Cas9 mRNA, sgRNAs, and/or donor DNAs were co-microinjected into zebrafish oocytes for gene knock-out or knock-in experiments. The efficiency of gene knock-in was successfully improved by 49.6% in the *in vitro* oocyte injection groups compared to 26% in the one-cell embryos groups. The efficiency of gene knock-out was enhanced by 94.4%, 88.9%, 91.1%, 90.0%, and 93.3% in the *in vitro* oocyte injection groups compared to 86.7%, 18.9%, 32.2%, 33.3%, and 40.7% in the one-cell embryos injection groups for the *mc4r*, *mpv17*, *mstna*, *mc3r*, and *mrap2b* genes, respectively. Moreover, the efficiencies of germline transmission in the offspring with *mc4r* and *mpv17* mutations were 96.7% and 91%, which were significantly higher than for the common CRISPR/Cas9 system (70% and 35.2%) (Xie et al., 2016). Therefore, *in vitro* oocyte injection may be an alternative to one-cell embryo injection to improve the efficiency of genome editing.

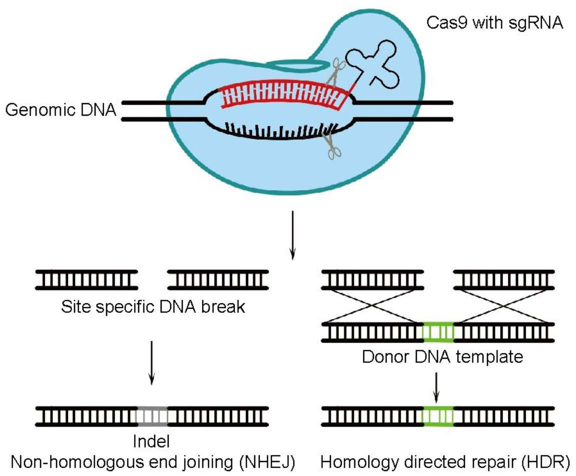

Figure 1 Schematic diagram of CRISPR-Cas9-mediated genome editing. Cas9 is guided by an sgRNA to induce a double-strand DNA break (DSB) at a desired genomic locus. The DSB can be repaired by NHEJ causing random insertion or deletion (indel) mutations or by HDR using a donor DNA template, enabling the introduction of desired sequence changes for precise genome editing purposes.

Lance array nanoinjection with Cas9 and sgRNAs

Lance array nanoinjection took advantage of a microfabricated silicon chip to physically and electrically deliver genetic material (*Sp*Cas9 and sgRNA) to large numbers of target cells. Sessions et al. generated an isogenic cell line containing a single copy of enhanced green fluorescent protein (EGFP) by cloning the coding sequence of EGFP into pCDNA5/FRT and then introducing this plasmid into HeLa/FRT cells in the presence of Flip recombinase. The HeLa/FRT cells expressed 99% GFP after selection by hygromycin and the GFP+/FRT HeLa cell line was obtained. The CRISPR-*Sp*Cas9 plasmid containing sgRNA targeting the N-terminus of EGFP was constructed and transferred into the GFP+/FRT HeLa cell line by lance array nanoinjection to knock-out the *EGFP* gene. This transfection technology achieved highly efficient genome editing after three injections at a current control setting of 4.5 mA, reaching a median level of 93.77% *EGFP* gene disruption (Sessions et al., 2016). Therefore, lance array nanoinjection may be a viable alternative to non-viral and viral delivery systems for CRISPR-Cas9 technology in the genome editing field.

Electroporation with Cas9 and sgRNAs

*Sp*Cas9 plasmid guiding by *CDK11* sgRNA was used to effectively silence endogenous *CDK11* in osteosarcoma cell lines using electroporation transfection. *CDK11* expression in KHOS cells was repressed by 8–12-fold at 48 h and 6–12-fold at 72 h. Similarly, *CDK11* expression in U-2OS was suppressed by 3–5-fold at 48 h and 7–15-fold at 72 h. The proliferation, viability, migration, and invasion activities were markedly reduced by CRISPR-Cas9-mediated *CDK11* knock-out (Feng et al., 2015). Su et al. demonstrated targeted gene knock-out of programmed death-1 (*PD-1*) via electroporation of sgRNA and *Sp*Cas9-encoding plasmids into primary human T cells, with mutation sizes ranging from −86 to +51 at an efficiency of 61.9% for sg1, 52.6% for sg2, 40% for sg3, 52.6% for sg4, 47.6% for sg (1+2), and 38.9% for sg (3+4). As a result, *PD-1* expression was significantly reduced, which upregulated IFN-γ production and enhanced cytotoxicity in cancer cells. The authors described for the first time a non-viral-mediated approach for reprogramming primary human T cells by disruption of *PD-1* (Su et al., 2016). Similarly, human primary T cells were electroporated with *Sp*Ca9 protein, sgRNA, and an HDR template to precisely target nucleotide replacements in T cells at *CXCR4* and *PD-1* loci with up to ~20% efficiency (~22% was achieved with 50 pmol and ~18% with 100 pmol of HDR template), leading to enhanced T cell effector function (Schumann et al., 2015). To produce genetically modified pigs, electroporation was exploited to introduce *Sp*Cas9 protein and sgRNA into *in vitro*-fertilized pig zygotes. Gene editing by electroporation of *Sp*Cas9 protein resulted in efficient targeted gene disruption (90%) and may be useful in the genetic modification of pigs (Tanihara et al., 2016).

Improved electroporation using Nucleofector technology enabled CRISPR-Cas9/sgRNA substrates delivery not only into the cytoplasm, but also through the nuclear membrane and into the nucleus. Therefore, this technology has been used for CRISPR-Cas9 system delivery by many scientists. Nucleofector Kit V were bound to the *Sp*Cas9-sgRNA plasmid targeting *ASXL1* and ssDNA template and then the ternary complexes were used to correct leukemia cells (KBM5) with *ASXL1* mutation *in vitro*. *ASXL1* gene expression was restored in 0.46%–2% of *ASXL1* mutation-corrected KBM5 cells after CRISPR-Cas9 genome editing *in vitro*. Mice xenografted with mutation-corrected KBM5 cells showed significantly longer survival than uncorrected xenografts *in vivo* (Valletta et al., 2015). Human primary CD4$^+$ T cells and CD34$^+$ hematopoietic stem and progenitor cells were transfected with *CCR5*- or *B2M*-specific gRNA/*Sp*Cas9 encoding plasmids with respective Nucleofector kits using a cell-specific Nucleofector program with a Nucleofector II device. The results demonstrated that CRISPR/Cas9 ablated 34% *B2M* in CD4$^+$ T cells and 42% *CCR5* in CD34$^+$ hematopoietic stem and progenitor cells with minimal off-target mutagenesis (Mandal et al., 2014). The 4D-Nucleofector X Kit transferred *Sp*Cas9, transcribed crRNA, and tracrRNA into K562 cells to analyze the off-target effects of CRISPR/Cas9-derived RNA-guided endonucleases and nickases (Cho et al., 2014). Nucleofections were performed using the DN-100 program on a Lonza 4-D Nucleofector with the SE Cell Line Kit. Cells were co-transfected with *Sp*Cas9 plasmids containing amino acid substitutions and the sgRNA plasmid to achieve high-fidelity *Sp*Cas9 (*Sp*Cas9-HF1), which rendered off-target events undetectable and maintained on-target activities (Kleinstiver et al., 2016). Fibroblasts and pluripotent stem cells were transfected with *Sp*Cas9 protein/transcribed sgRNA or *Sp*Cas9 encoding the plasmid/sgRNA-expressing plasmid using the respective Nucleofector kits. *Sp*Cas9 protein cleaved up to 79% of chromosomal DNA nearly immediately after delivery and was degraded rapidly in cells. The authors suggested that Cas9 protein, rather than the Cas9 gene, prevented the persistent effect on the genome and reduced off-target effects (Kim et al., 2014). Thus, electroporation transfection is an effective delivery approach for the CRISPR-Cas9 system and has been widely adopted in *in vitro* studies of genome editing.

Hydrodynamic injection with Cas9 and sgRNAs

Lin et al. showed that hepatitis B virus (HBV)-specific sgRNA/*Sp*Cas9 expression plasmids introduced via hydrodynamic injection disrupted and eliminated the intrahepatic HBV genome with a 5% mutagenesis rate by T7E1 and 27% mutagenesis rate by clonal sequencing *in vivo*, ultimately reducing the levels of serum HBV surface antigens in an HBV persistent mouse model (Lin et al., 2014a). Xue et al. directly disrupted tumor suppressor genes and induced point mutations in oncogenes in the adult mouse liver using the CRISPR/Cas9 system via hydrodynamic injection, resulting in compound *Pten* and *p53* indels at low frequency, which was sufficient for generating multifocal tumors in the mouse liver (Xue et al., 2014). For hydrodynamic liver injection, an *Sp*Cas9-sgRNA expression plasmid and an ssDNA donor template were injected via the tail vein into *Fah*$^{mut/mut}$ (fumarylacetoacetate hydrolase, *Fah*) mice. Delivery of

CRISPR/Cas9 system components by hydrodynamic injection resulted in initial expression of wild-type *Fah* protein in approximately 1/250 liver cells. Yin et al. demonstrated *Sp*Cas9-meditated correction of the *Fah* mutation in hepatocytes in the mouse model of the human disease hereditary tyrosinemia (Yin et al., 2014). The CRISPR-Cas9 system can be used for genome editing *in vivo* via hydrodynamic injection.

NON-VIRAL VECTORS FOR CRISPR-CAS9 SYSTEM

Liposomes

sgRNA can be cloned into Cas9-expressing plasmids (pX260, pX330, pX458, pX459, among others) and HDR templates can be constructed into plasmid vectors. To generate −45 *Nanog* super-enhancer deleted embryonic stem cell clones, Lipofectamine 2000 was used to deliver a *Sp*Cas9-sgRNA plasmid targeting the −45 enhancer and HDR vector to co-transfected embryonic stem cells. This demonstrated the functionality of the −45 enhancer in the regulation of both nearest neighbor genes, *Nanog* and *Dppa3* (Blinka et al., 2016). Lipofectamine 2000 transferred p*Sp*Cas9s and sgRNAs targeting *hBAX*, *p21*, and *E-cadherin* into bladder cancer cells. This CRISPR-Cas9 system effectively inhibited cancer cell growth, induced cancer cell apoptosis, and decreased cell motility by activating these tumor suppressors in bladder cancer cells *in vitro* (Liu et al., 2014). Transfections were performed with Lipofectamine 2000 to screen for enhanced specificity *Sp*Cas9 (e*Sp*Cas9) or a Cas9 orthologue from *Staphylococcus aureus* (*Sa*Cas9, e*Sa*Cas9). For screening of the e*Sp*Cas9 or e*Sa*Cas9, Cas9 plasmids with point mutations and sgRNA, plasmids were added to cells for transfection. e*Sp*Cas9 or e*Sa*Cas9 reduced off-target effects and retained robust on-target cleavage (Slaymaker et al., 2016). Lipofectamine 2000 delivered an intein-*Sp*Cas9 or wild-type *Sp*Cas9 expression plasmid and sgRNA expression plasmid into human cells for evaluation of the specificity of small molecule-triggered *Sp*Cas9 protein. In human cells, 4-hydrotamoxifen conditionally active *Sp*Cas9 modified the target genomic sites with up to 25-fold higher specificity than wild-type *Sp*Cas9 (Davis et al., 2015). Complexes of Lipofectamine 3000 (or Lipofectamine 2000) and HBV-specific *Sp*Cas9/sgRNAs remarkably decreased production of the HBV core and surface proteins in Huh-7 cells transfected with an HBV-expression vector (Lin et al., 2014a). Lipofectamine LTX was utilized to deliver *Sp*Cas9-sgRNAs plasmids to inactivate HBV by simultaneously targeting multiple HBV domains *in vitro* (Sakuma et al., 2016). Lipofectamine LTX was used to assess on-target and off-target indel mutations induced by *Sp*Cas9 or *Sp*Cas9-D10A nickase expression plasmids and truncated sgRNA expression plasmids. In addition, Lipofectamine LTX was used to evaluate the frequencies of precise alterations introduced by HDR with ssDNA templates. Truncated sgRNA effectively decreased undesired mutagenesis at some off-target sites without sacrificing on-target genome editing efficiencies. Furthermore, the use of truncated gRNAs may reduce off-target effects induced by pairs of *Sp*Cas9 variants that nick DNA (paired nickases) (Fu et al., 2014). Liposomal formulations, including Lipofectamine RNAiMAX, Lipofectamine 2000, Lipofectamine LTX, and SAINT-Red (containing a synthetic pyridinium-based cationic lipid), were more effective functional delivery agents for multiple *Sp*Cas9 versions than the cationic lipid DOTAP and EZ-PLEX (peptide-based nucleic acid delivery agent). Wild-type *Sp*Cas9/sgRNA liposomal delivery modified genomes with greater specificity than plasmid DNA transfection. Furthermore, this approach efficiently delivered Cre recombinase and *Sp*Cas9:sgRNA complexes into the inner ear *in vivo*, achieving 90% Cre-mediated recombination and 20% *Sp*Cas9-mediated genome modification in the hair cells of mice (Zuris et al., 2015).

Nanoparticles

FuGene6 was complexed with HPV-18 E6- or E7-specific *Sp*Cas9-sgRNA expression plasmids, and HeLa cells were co-transfected *in vitro* with Fugene6-Cas9 complexes, which induced cleavage of the HPV genome and introduction of inactivating indel mutations into the E6 and E7 gene. E6 and E7 gene knock-out inhibited cervical tumor growth and reversed the malignant phenotype (Kennedy et al., 2014). FuGene HD-transfected cells with sgRNA plasmids or *sp*Cas9 plasmid and 27%–45% indels were induced by T7E1 assay according to the different GC contents of sgRNAs. The results indicate that the genomic sites were effectively cleaved by this CRISPR/Cas9 system (Lin et al., 2014b).

A cationic material poly(CBA-ABOL) was used to condense dCas9-VP64 (*Sp*Cas9 was mutated at catalytic residues D10A and H840A and genetically fused with a C-terminal VP64 acidic transactivation domain) and four sgRNA expression plasmids, and endogenous genes encoding key regulators of cell fate were activated *in vitro* (Adler et al., 2012; Perez-Pinera et al., 2013). To enhance the selectivity of vaccinia virus to cancer cells in oncolytic virotherapy, CRISPR-Cas9 was used to delete the thymidine kinase region in the genome of vaccinia virus. An sgRNA expression plasmid was co-transfected with *Sp*Cas9 into CV-1 (monkey kidney fibroblast) cells using Effectene transfection reagent. Next, a repair donor template was transfected into cells that had been infected with 0.01 pfu cell^{-1} of backbone virus. The thymidine kinase gene was efficiently replaced (~90%) with a red fluorescent protein gene using the CRISPR-Cas9 system (Yuan et al., 2015a; Yuan et al., 2015b).

Similarly, polyethyleneimine (PEI) mixed with herpes simplex virus (HSV)-specific sgRNA/*Sp*Cas9 constructs and pCIneo-CD8 plasmid (expressing human CD8A for sorting) were added to HEK 293T cells. $CD8^+$ cells in the transfectants were isolated by flow cytometry and infected with HSV-1. A donor template for repairing or knock-in was retransfected using PEI to generate revertant or knock-in viruses. Not only gene-ablated HSV (over 50%), but also gene knock-in HSV (approximately 10%) were generated via this method (Suenaga et al., 2014).

A polyamine transfection reagent (TransIT-LT1) was used to transfect HEK293 cells with wild-type *Sp*Cas9 or human codon-optimized *Fok* I-dCas9 nuclease plasmid, sgRNA expression plasmid, and tdTomato expression plasmid, and NHEJ-mediated mutagenesis was analyzed after transfection. A total of 75%–90% of the target gene was disrupted by this Cas9 system. Moreover, the *Fok* I-dCas9 fusion protein with high efficiency showed higher specificity than wild-type *Sp*Cas9, while off-target mutations were reduced to undetectable levels (Tsai et al., 2014).

A multi-component DNA transfection reagent (X-tremGENE HP) formed a complex with *Sp*Cas9 variants expressing sgRNA and/or donor plasmid, and then the complex was transported into cells and the targeting efficiency of *Sp*Cas9 variants was determined. Approximately 23% indels were observed in the transfected cells, indicating that the CRISPR-Cas9 system was effective in cells (Truong et al., 2015).

A cationic polymer transfection reagent (TurboFect) encapsulated *As*Cpf1 from *Acidaminococcus* sp., *Lb*Cpf1 from *Lachnospiraceae bacterium*, *St1*Cas9 from *Streptococcus thermophiles* LMD-9, *Sp*Cas9, or *Sa*Cas9 plasmid together with their cognate crRANs to transfect Neuro-2a mouse neuroblastoma cells to induce HDR. The results suggest that *As*Cpf1 or *Lb*Cpf1 efficiently generated double-strand breaks and induce 24% or 15% HDR, which was similar to the most frequently used orthogonal Cas9 (13% for *Sa*Cas9 or 9% for *St1*Cas9) (Tóth et al., 2016).

Cell-penetrating peptide-mediated delivery of *Sp*Cas9 protein and sgRNA

Ramakrishna et al. postulated that introduction of a cell-penetrating peptide (CPP) into the *Sp*Cas9 protein would enable its direct delivery into cells. Genetically fusing *Sp*Cas9 to a CPP consisting of four Gly, nine Arg, and four Leu made it difficult to obtain purified protein in suitable quantities. Therefore, a Cys residue at the C-terminus was added by minimizing the genetic modification of *Sp*Cas9. A primary amine (-NH_2) residue in maleimide-linked CPP reacted with free SH residue in the C-terminal cysteine of *Sp*Cas9 to form a CPP-*Sp*Cas9 conjugation via a thioether bond. Additionally, CPP mixed with sgRNA formed condensed, positively charged nanoparticles (CPP-sgRNA complex) at appropriate weight ratios. Then human cells including embryonic stem cells, dermal fibroblasts, HEK 293T cells, HeLa cells, and embryonic carcinoma cells were treated by CPP-*Sp*Cas9 conjugation and CPP-sgRNA complex either sequentially or simultaneously. CPP-mediated delivery of *Sp*Cas9 and sgRNA generated showed gene disruption (8.7%–14%) with reduced off-target effects. CPP-mediated delivery may facilitate CRISPR/Cas9 system-directed genome editing (Ramakrishna et al., 2014; Suresh et al., 2017).

In conclusion, synthetic and commercial cationic materials can bind CRISPR-Cas9 vectors to form cationic materials/Cas9 complexes, which deliver the CRISPR-Cas9 system into cells and induce indel mutations or HDR at the target site. Generally, non-viral vector/Cas9 complex systems can be used for genome editing studies *in vitro*, and non-viral vectors require further improvements to deliver the CRISPR-Cas9 system *in vivo*.

VIRAL VECTORS FOR CRISPR-CAS9 SYSTEM

Retroviruses

Retrovirus coding for *Sp*Cas9 was used to transduce HeLa cells and generate HeLa cells that stably expressed RNA-guided endonuclease *Sp*Cas9 (Tao et al., 2016). Retrovirus expressing *Sp*Cas9 and sgRNA transduced primary mouse B cells and induced high levels of class-switch recombination in mouse B cells activated *in vitro* by anti-CD40 antibody and interleukin-4. The CRISPR-Cas9-retroviral vector switched AID-deficient B cells from IgM to IgG1 (Cheong et al., 2016). Katanin P60 subunit A-like 2 (*Katnal2*) is an understudied autism-linked gene and presumptive microtubule-severing ATPase in which mutations have been associated with autism through whole-exome sequencing. Williams et al. designed and constructed a retrovirus expressing GFP, *Sp*Cas9, and two sgRNAs flanking the start codon for the major predicted transcript variants 1, 2, and 4 to knock-out mouse *Katnal2* expression. This retrovirus introduced indels into the region near sgRNA1 and sgRNA2 into approximately 93% (14/15) of N2A cell clones that had been infected with the retrovirus. Furthermore, the retrovirus caused *Katnal2* deletion in the mouse, decreasing the dendritic arborization of developing neurons. Therefore, retroviruses are useful vectors for CRISPR-Cas9 technology (Fricano-Kugler et al., 2016; Williams et al., 2016).

Adenovirus

An adenovirus-expressing vector of *Sp*Cas9 and sgRNAs was utilized to correct *DMD* in *mdx* mice. Adenovirus-mediated transduction of *Sp*Cas9/sgRNA corrected the gene mutation and restored dystrophin expression in *mdx* mice after intramuscular injection (Xu et al., 2016). An adenoviral vector encoding *Sp*Cas9 and sgRNA transduced the CRISPR-Cas9 system into transformed and non-transformed cells and induced effective gene disruption. In addition, the frequencies of gene disruption were 18%–65% in various cell types (including dividing and quiescent primary cells) (Maggio et al., 2014). An E1/E3-deleted adenovirus vector co-expressing *Sp*Cas9 and sgRNA targeting mouse/human Pten gene was packaged (Ad.sgPten). In both mouse (KP) and human (HEK293T) cells, Ad.sgPten infection resulted in indel mutations in *Pten* as evidenced by the Surveyor assay. Furthermore, Ad.sgPten delivered the *Sp*Cas9-mediated *Pten* gene editing system to the mouse liver and the total indel frequencies were 14.8% and 22.8% in the two mice examined (Wang et al., 2015). Therefore, adenovirus is an efficient vector for *in vivo* delivery of CRISPR-Cas9 technology.

Lentiviruses

Lentiviral vectors were used to transduce HBV-specific sgRNAs and *Sp*Cas9 into both a chronic HBV infection cell model and *de novo* HBV infection, and approximately 76% of indels were observed in the transduced samples. Cas9/sgRNA combinations specific for HBV reduced total viral DNA levels by up to ~1,000-fold and HBV cccDNA levels by up to ~10-fold, as well as mutationally inactivated most residual viral DNA (Kennedy et al., 2015). *Sp*Cas9 and sgRNA were packaged into two lentiviral vectors, which were used to transduce cells for targeted double-strand break introduction. Next, the transduced cells were transfected with a plasmid donor for HDR. Efficient and precise genome editing was achieved using the lentiviral CRISPR/Cas9 system and the efficiency of HDR-mediated genome editing was up to 59.3% when an NEHJ inhibitor was used for Scr7 treatment (Maruyama et al., 2015). The lentiviral expression vector for *Sp*Cas9 and sgRNA used to modify specific genomic loci provided a new method for evaluating gene function on a genome-wide scale. Shalem et al. showed that the lentiviral delivery of a genome-scale CRISPR-Cas9 knock-out (GeCKO) library targeting 18,080 genes with 64,751 unique sgRNAs enabled both negative and positive selection screening in human cells. This screen successfully yielded high-ranking candidate genes that included two previously validated genes and four novel hits (Shalem et al., 2014). Lentiviral libraries expressing sgRNAs targeting 19,052 genes, with six sgRNAs per gene, transduced HeLa cells that stably expressed RNA-guided endonuclease Cas9 (Tao et al., 2016). The *MCL-1* gene was deleted in human Burkitt lymphoma cells using a lentiviral CRISPR-Cas9 platform, which resulted in the apoptosis of Burkitt lymphoma cells at a high frequency (80% mutation rate). Moreover, in a human Burkitt lymphoma xenograft model *in vivo*, Aubrey et al. observed dramatic tumor regression or impaired growth by repeated induction of sgRNA, which was expressed by the lentiviral CRISPR-Cas9 platform (Aubrey et al., 2015; Yi and Li, 2016).

Adeno-associated virus (AAV) vectors

AAV vectors are attractive vehicles because of their high infection efficiency, low immunogenic potential, reduced oncogenic risk from host-genome integration, and broad range of serotype specificity (Ran et al., 2015; Truong et al., 2015). AAV can package *Sp*Cas9 (~4.2 kb) and sgRNA with a promoter (~0.3 kb) in a single vector, but leaves little room for customized expression and control elements because of the restrictive packaging capacity of AAV (~4.5 kb, excluding inverted terminal repeats) (Ran et al., 2015). Truong et al. took advantage of the structural knowledge related to *Sp*Cas9 and created a split-intein-mediated split-*Sp*Cas9 trans-splicing system, which allowed the coding sequence of *Sp*Cas9 to be distributed on a dual-AAV vector and reconstituted post-translationally. The genome editing activity of the split-intein system was similar to that of wild-type *Sp*Cas9. This strategy was suitable for *Sp*Cas9^{D10A} nickase. Moreover, the dual-AAV system increased the efficiency of HDR. Intein-mediated split-*Sp*Cas9 could be packaged and delivered via AAV and its nuclease activity could be reconstituted efficiently in cells (Truong et al., 2015). A rationally designed truncated form of *Sp*Cas9 (~4.0 kb) is shorter than wild-type *Sp*Cas9 (~4.2 kb), but the truncation version of *Sp*Cas9 exhibited reduced activity (Nishimasu et al., 2014). *St1*Cas9 that was ~3.4 kb in size had a narrow genomic target with a complex PAM sequence (NNAGAAW) (Cong et al., 2013; Garneau et al., 2010). *Sa*Cas9 (~3.2 kb) generated indels with efficiencies comparable to those of *Sp*Cas9. Therefore, the small *Sa*Cas9 and its sgRNA expression cassette were incorporated into an AAV8 vector and targeted the cholesterol regulatory gene *Pcsk9* in the mouse liver. Within one week of injection, Ran et al. observed >40% gene modification, accompanied by significant reductions in serum *Pcsk9* and total cholesterol levels. The results indicated that AAV-*Sa*Cas9 system-mediated *in vivo* genome editing is efficient and specific (Ran et al., 2015). Recombinant AAV9 was used to systemically deliver *Sp*Cas9 or *Sa*Cas9 and sgRNAs targeting the *DMD* (dystrophia) gene to muscle tissues of *mdx* mice, and *DMD* mutation in these *mdx* mice was efficiently edited and dystrophin expression was partially restored in the mouse model of muscular dystrophy (Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016). A dual-AAV system that packaged *Sp*Cas9 and sgRNA expression cassettes in two separate viral vectors (AAV1/2) has been harnessed to deliver the CRISPR/Cas9 system into adult mouse brain by stereotactic injection. The dual-AAV system could edit single (*Mecp2*) and multiple (*Dnmt1*, *3a*, and *3b*) epigenetic targets *in vivo* (Mentis, 2016; Swiech et al., 2015). Therefore, AAV is the most promising vector based on the CRISPR-Cas9 technique for human gene therapy.

COMBINED NON-VIRAL AND VIRAL DELIVERY

Non-viral delivery of Cas9 would allow for short-term expression and complete removal from the body, which may avoid immune responses and off-target effects due to long-term expression of Cas9 *in vivo* (Figure 2) (Yao et al., 2015). Lipid-like materials C12-200/cholesterol/C14PEG2000/DOPE/arachidonic acid were used to encapsulate *Sp*Cas9 mRNA (~4.5 kb) to prepare lipid nanoparticles (NanoCas9) for non-viral delivery. An AAV2/8 serotype vector with a U6-sgRNA expression cassette and HDR template (AAV-sgRNA-HDR) was produced to target

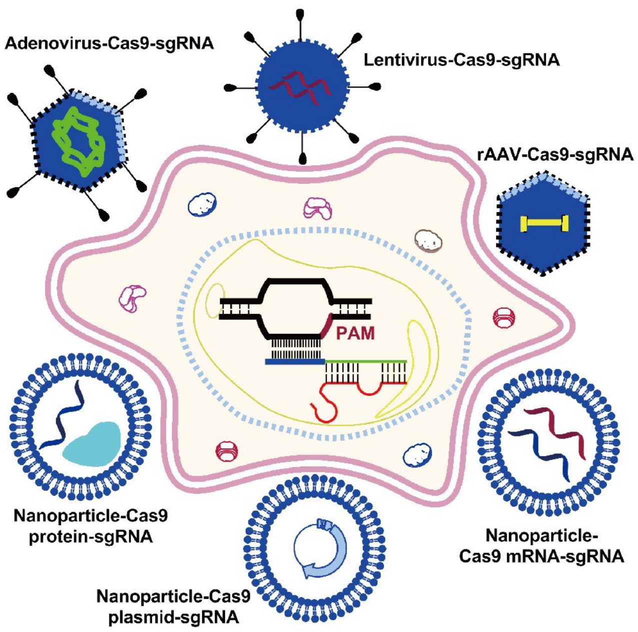

Figure 2 Delivery vectors for CRISPR-Cas9 systems. Human codon-optimized Cas9 and sgRNA sequences were packaged into a viral vector (e.g., adenovirus, rAAV, lentivirus) for genome editing. Cas9 protein, mRNA of Cas9 and sgRNA, or a plasmid encoding Cas9 and sgRNA was incorporated into a nanoparticle to formulate a nano-Cas9 complex for non-viral delivery.

and repair *Fah* mutation in hepatocytes in an $Fah^{mut/mut}$ mouse. NanoCas9 and AAV-sgRNA-HDR were introduced in 8–10-week old $Fah^{mut/mut}$ mice via tail vein injection. This combined non-viral and viral delivery yielded *Fah*-positive hepatocytes by correcting the causative *Fah*-splicing mutation and relieved disease symptoms such as weight loss and liver damage. Furthermore, the *in vivo* off-target lesion rate was low for viral sgRNA in conjunction with non-viral mRNA delivery of *Sp*Cas9. The efficiency of correction was >6% of hepatocytes after single administration, suggesting the potential application of combined non-viral and viral delivery-based therapeutic genome editing for a range of diseases (Yin et al., 2016).

CONCLUSION AND FUTURE PERSPECTIVES

The features of all delivery systems discussed for CRISPR-Cas9 technology and their applications in the biomedical field are summarized in Table 1 (Liu and Shui, 2016; Wang et al., 2016). Commercially and commonly non-viral vectors can deliver the CRISPR-Cas9 system into cells *in vitro* to edit target sites in the genome. Cas9 protein or transient expression of Cas9 via non-viral delivery avoids an immune response caused by persistent expression of Cas9 and reduces off-target effects *in vivo*. Compared to non-viral vectors, viral vectors transfer the CRISPR-Cas9 system into target tissue *in vivo*, generate double-strand breaks at the target site, and result in point mutation via NHEJ or gene repair via HDR. Viral vectors exhibit clear advantages over non-viral vectors, not only in gene knock-out but also in gene knock-in. However, persistent expression of Cas9 via viral vectors may induce immune responses and off-target effects, and thus must be further improved. In the future, sgRNA and donor template may

Table 1 Non-viral and viral vectors for CRISPR-Cas9 system and their applications in the biomedical field

Delivery methods	Advantages	Disadvantages	Applications
Microinjection	High efficiency *in vitro*	Low-throughput	Genome editing for oocytes or embryos; generation of model animals
Electroporation	High transfection efficiency *in vitro*	Cytotoxicity, difficult for *in vivo* use	Genome editing for various cell types *in vitro*
Hydrodynamic injection	Feasible for *in vivo* gene editing in small animals	Low efficiency, difficult for clinical use	Gene function study *in vivo*
CPP	Low off-target effects	Low efficiency, immunogenicity, difficult for *in vivo* use	Genome editing for cells *in vitro*
Cationic vectors	Easy to produce, large packaging capacity	Low efficiency	Genome editing for various cell types *in vitro*; gene therapy for cancer, HBV, genetic diseases, etc.
Retrovirus	High efficiency *in vivo*, integrating target gene into host cell genome	Insertional mutagenesis, oncogene activation	Gene therapy for cancer, genetic diseases, etc.
Lentivirus	High efficiency, high throughput *in vitro* and *in vivo*	Prone to rearrangements of cargo genes, liable to transgene silencing	Genomic screen and gene function study *in vitro* and *in vivo*
Adenovirus	High efficiency *in vivo*, high packaging capacity	Immunoreactivity, difficult to produce in large scale	Gene therapy for genetic diseases
AAV	High efficiency *in vivo*, non-pathogenic	Limited packaging capacity, high cost	Gene therapy for various genetic diseases

be constructed into a viral vector for persistent expression, and transient Cas9 can be delivered via a non-viral vector and multi-administered for effective DNA cleavage. Therefore, the combination viral vector with multi-administered non-viral vector may be an optimal approach for precise medicine based on CRISPR-Cas9 technology.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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