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COMMENTARY

CRISPR-Cas9 for *in vivo* Gene Therapy: Promise and Hurdles

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Owing to its easy-to-use and multiplexing nature, the genome editing tool CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats (CRISPR) associated nuclease 9) is revolutionizing many areas of medical research and one of the most amazing areas is its gene therapy potentials. Previous explorations into the therapeutic potentials of CRISPR-Cas9 were mainly conducted *in vitro* or in animal germlines, the translatability of which, however, is either limited (to tissues with adult stem cells amenable to culture and manipulation) or currently impermissible (due to ethic concerns). Recently, important progresses have been made on this regard. Several studies have demonstrated the ability of CRISPR-Cas9 for *in vivo* gene therapy in adult rodent models of human genetic diseases delivered by methods that are potentially translatable to human use. Although these recent advances represent a significant step forward to the eventual application of CRISPR-Cas9 to the clinic, there are still many hurdles to overcome, such as the off-target effects of CRISPR-Cas9, efficacy of homology-directed repair, fitness of edited cells, immunogenicity of therapeutic CRISPR-Cas9 components, as well as efficiency, specificity, and translatability of *in vivo* delivery methods. In this article, we introduce the mechanisms and merits of CRISPR-Cas9 in genome editing, briefly retrospect the applications of CRISPR-Cas9 in gene therapy explorations and highlight recent advances, later we discuss in detail the challenges lying ahead in the way of its translatability, propose possible solutions, and future research directions.

MECHANISMS AND MERITS OF CRISPR-CAS9 IN GENOME EDITING

Clustered regularly interspaced short palindromic repeats (CRISPR) associated nuclease 9 (CRISPR-Cas9) is a RNA-guided genome-editing tool derived from microbial adaptive immune defense system. It comprises a nuclease called Cas9 and a single guide RNA (sgRNA) that recognizes target DNA by Watson-Crick base-pairing[1–3] (**Figure 1a**,**b**). Guided by the sgRNA, Cas9 binds to the target loci adjacent to a protospacer adjacent motif and generates site-specific double-strand breaks[1–4] (**Figure 1b**). These double-strand breaks are subsequently repaired either by nonhomologous end-joining (NHEJ) or by homology-directed repair (HDR) upon the existence of a donor template. NHEJ is more efficient than HDR but is error prone and may produce indel mutations, whereas HDR can provide a precise gene modification[5,6] (**Figure 1c**).

Compared with conventional programmable nucleases such as zinc finger nuclease and transcription activator-like effector nuclease, the RNA-guided CRISPR-Cas9 possesses several advantages. First, CRISPR-Cas9 system is easier to design and simpler to use because targeting a new locus requires only the redesign of a sgRNA rather than synthesis of a new guiding protein as in zinc finger nuclease and transcription activator-like effector nuclease. Second, CRISPR-Cas9 is multiplexing in that multiple loci can be targeted simultaneously if multiple sgRNAs are provided.[2] Moreover, wide-type (wt)--Cas9 can be reprogrammed into catalytically inactive Cas9 (dead Cas9) that when fused to transcriptional modifiers such as VP16 can modulate target gene expression.[7,8]

Since its introduction into mammalian cells in 2013,[1,2] CRISPR-Cas9 has been applied to and has been revolutionizing many areas of medical research,[4,9,10] and the most amazing one is gene therapy explorations of human diseases.[11]

APPLICATIONS AND RECENT ADVANCES OF CRISPR-CAS9 IN GENE THERAPY EXPLORATIONS

Just several months after its introduction into mammalian cells,[1,2] CRISPR-Cas9 demonstrated its potentials in gene therapy by mutating HIV-1 to decrease its expression in human T cells.[12] Since then, much effort has been made to explore the therapeutic potentials of CRISPR-Cas9 in combating infections such as hepatitis B virus[13] and human papillomaviruses,[14] in correcting culprit mutations in monogenic diseases in model organisms,[15,16] and in inducing therapeutic or protective mutations in host cells.[17–19] However, these studies were conducted either in cells or animal germlines. As we

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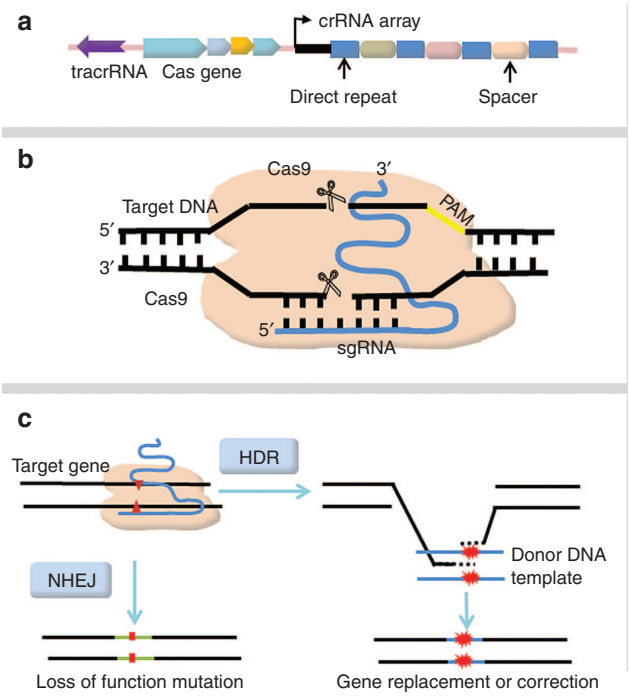

Figure 1 Schematic representation of CRISPR-Cas9-mediated genome editing. (**a**) Schematic of CRISPR locus (from *Streptococcus pyogenes*). (**b**) Site-specific DNA cleavage by nuclease Cas9 directed by complementary between a single guide RNA (sgRNA) and the target sequence upon the presence of a protospacer-adjacent motif (PAM) on the opposite strand. (**c**) The resultant double-strand breaks (DSBs) are subsequently repaired either by nonhomologous end-joining (NHEJ) or by homology-directed repair (HDR) upon the existence of a donor template. NHEJ is more efficient than HDR but is error prone and may produce indel mutations, whereas HDR can provide a precise gene modification.

know, germline manipulation is unfeasible in humans due to ethical concerns and *ex vivo* gene modification followed by transplantation is technologically challenging for daily clinical practice. Therefore, more clinical translatable methods to deliver therapeutic CRISPR-Cas9 components are still needed.

Recently, important progresses have been made on this regard. Three studies[20–22] published simultaneously in *Science*, for example, reported that CRISPR-Cas9 components delivered through intramuscular, intraperitoneal or intravenous injection corrected the culprit gene mutation in mouse models of Duchenne muscular dystrophy (DMD) and rescued the disease phenotype. DMD is a monogenic disease caused by mutations in the gene encoding a protein termed dystrophin that is necessary for muscle cell integrity. In mouse models of DMD generated by a nonsense mutation in exon 23 of *Dmd* gene, CRISPR-Cas9 targeting intron 22 and intron 23 of *Dmd* gene removed the disease-causing mutation in a proportion of muscle cells in neonatal or adult mice, resulting in restoration of dystrophin expression and muscle function.[20–22] Two other studies[23,24] published back-to-back in Nature Biotechnology demonstrated the efficacy of CRISPR-Cas9-mediated HDR for *in vivo* gene therapy through intravenous injection in mouse models of human hereditary liver diseases. Compared with previous studies in cell lines or animal germlines,[12–19] these five recent studies[20–24] represent a significant step forward because they have demonstrated the ability of CRISPR-Cas9 for *in vivo* gene therapy delivered by methods that are potentially translatable to human use.[20–24] Moreover, the viral vectors used in these studies are adeno-associated viruses (AAVs), the safety and efficacy of which have been tested in clinical trials[25] and has recently been approved for clinic use.[26] These exciting results highlighted the promise of CRISPR-Cas9 as a gene therapy tool in the future.

CHALLENGES AND POSSIBLE SOLUTIONS

Despite recent exciting advances, there are still many challenges to overcome for the final applications of CRISPR-Cas9 to clinical gene therapy, such as the specificity and efficacy of CRISPR-Cas9 in therapeutic genome editing, efficacy and translatability of *in vivo* delivery methods, and potential immunogenicity of CRISPR-Cas9 and the delivery vehicles.

Specificity of CRISPR-Cas9

One of the major hurdles to the clinical translation of CRISPR-Cas9 is its off-target effects, which may lead to uncontrollable and unpredictable consequences including malignant transformation. Broadly speaking, off-target effects include off-target editing, off-target binding and other functional consequences imposed by the introduction of CRISPR-Cas9 into cells. Although recent studies[20–24] detected no or only minimal off-target editing in predicted sites following *in vivo* gene therapy in mice, genome-wide unbiased methods such as ChIP-seq[27] (chromatin immunoprecipitation followed by sequencing) and Digenome-seq[28] should be harnessed to offer a more comprehensive profile of off-target editing because they may occur beyond the predicted sites. Moreover, off-target binding events that may interfere with gene transcription and other functional consequences imposed by CRISPR-Cas9 introduction should also be precisely profiled and put under control.

Much effort has been made to reduce the off-target effects, such as modifying Cas9 construction,[29] optimizing sgRNA design[30] and the nickase Cas9 strategy.[31] For *in vivo* gene therapy, a "hit-and-run" strategy by delivering Cas9 protein instead of *Cas9* gene[32] or by nonviral delivery[24] of Cas9 mRNA is preferred because it allows for only transient Cas9 existence, avoiding persistent Cas9 exposure and thus reducing off-target effects. Due to the potential devastating nature of off-target effects, they must be studied thoroughly and tested in large-animal models before applied to humans.

Efficacy of HDR-mediated gene correction

HDR-mediated gene correction has broader application spectrum than NHEJ-mediated gene deletion or inactivation because there are far more diseases whose treatments necessitate precise gene correction than those requiring only culprit gene deletion or inactivation. In the case of hereditary tyrosinemia type I, for example, the disease-causing point mutation (G→A) in *FAH* gene needs to be rectified to cure the disease.[24] However, HDR is less efficient than NHEJ in that the former needs a donor template and does not take place in postmitotic adult tissues. Therefore, it remains challenging

to improve the efficacy of HDR to levels that are sufficient for gene therapy. Many methods have been reported to increase HDR efficacy, such as rational design of single-stranded DNA donors,[33] inhibition of NHEJ pathway,[34,35] and increasing the extent of similarity between the donor templates and the double-strand break sites.[36]

Another challenge imposed by the need of correcting rather than deleting culprit mutations is that the variations in mutational patterns among individuals of the same disease may necessitate patient-specific design of sgRNAs and donor templates. This personalized requirement poses a big challenge to the scale production of CRISPR-Cas9 gene therapy drugs in the future.

Efficiency, specificity, and translatability of *in vivo* delivery methods

In vivo gene therapy with CRISPR-Cas9 involves efficient and tissue-specific delivery of CRISPR-Cas9. In the vast majority of *in vivo* studies, therapeutic CRISPR-Cas9 components were delivered via viral vectors, especially AAVs. However, AAVs have some limitations. First, the cargo capacity of AAVs is limited (~4.7 kb only). Second, the tissue tropism of AAVs is restricted to a few organs such as liver, muscle, brain, and eye.[37] And third, to increase tissue-specific therapeutic effects and in the meanwhile to reduce side effects in other tissues, the tissue specificity of AAVs remains to be improved to control the tissue distribution of CRISPR-Cas9. Engineering AAV to increase its genetic cargo[38] or to increase its tropism[39] for target tissue may represent promising ways to address these challenges. For example, by incorporating into AAV capsid the ligands specific to human epidermal growth factor receptor 2 that is overexpressed at the surface of tumor cell, Munch and colleagues[39] increased the tropism of AVV for tumor cells by ~20-fold *in vivo*.

Another challenge is the translatability of the delivery methods. Although *in vivo* delivery methods used in recent studies[20–24] (intramuscular, intraperitoneal or intravenous) are potentially translatable, there are still many uncertainties in their translatability. For example, the number of intramuscular injections that is required to treat a human might be too big to be practical for clinical use, and the translatability of intraperitoneal injections form mouse to humans is quite questionable. As for intravenous injection, it is certainly a clinically-relevant approach, but one should keep in mind that an enormous dose of vector would be required to reach anything close to the desired efficiency. Moreover, it remains unclear whether these delivery methods work effectively in humans given the huge difference in body size and the many other genetic and morphological differences between humans and rodents.

Immunogenicity of CRISPR-Cas9 and delivery vehicles

Possible host immune responses triggered by Cas9 proteins or delivery vehicles[40] represent another layer of hurdles to the *in vivo* therapeutic applications of CRISPR-Cas9. Immunogenicity of viral vectors is a common problem encountered in gene therapy endeavors,[40] and Cas9 proteins or peptides are also of potentially immunogenicity given their bacterium origin.

Host immune responses may attenuate therapeutic effects and cause side effects, thus should be minimized or circumvented. Developing nonviral vectors such as nanoparticle- and lipid-based vectors[24,41] may represent a promising way to circumvent the immunogenicity of viral vectors. Humanizing Cas9 protein is a potential strategy to minimize the immunogenicity of Cas9 peptides. Ways to reduce the immunogenicity of CRISPR-Cas9 components and delivery vehicles are interesting areas for future researches.

Fitness of edited cells

Therapeutic genome editing by CRISPR-Cas9 may alter the fitness of edited cells, which in turn can affect the efficacy and duration of gene therapy. In cases where therapeutic genome editing renders a growth advantage, the number of edited cells needed to rescue the disease phenotype is relatively small and therapeutic efficacy is easier to gain and sustain. In a study in which CRISPR-Cas9 was used to correct disease-causing mutation in a mouse model of hereditary tyrosinemia,[15] for example, only 0.25% of liver cells were initially genetically corrected, but 33 days later, the proportion of genetically corrected cells reached 33.5%, which was sufficient to rescue the disease phenotype.

On the contrary, there are also cases in which therapeutic genome editing renders a growth disadvantage. If we use CRISPR-Cas9 to inactivate oncogenes in cancer cells, for example, the genetically edited cells will be out competed by their unedited counterparts quickly because the latter retain malignancy and thus possess a growth advantage over the former. As a result, repeated episodes of treatment and pretty high editing efficiencies would be needed to be therapeutic, which is rather challenging and beyond the capacity of current CRISPR-Cas9 technologies.

CONCLUDING REMARKS

In conclusion, recent success in the *in vivo* gene therapy explorations in model animals has highlighted the promise of CRISPR-Cas9 as a gene therapy tool for genetic diseases. For the final application of CRISPR-Cas9 to the clinic, however, there are still many hurdles to overcome, such as off-target effects, HDR efficacy, efficiency and specificity of delivery vehicles, translatability of *in vivo* delivery methods, immunogenicity of delivery vehicles and Cas9 peptides, and fitness of edited cells. With the rapid advances in CRISPR technology, we can optimistically anticipate these hurdles to be overcome in the foreseeable future to pave the way for the final application of CRISPR-Cas9 to human gene therapies.

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