Gene Therapy (2016) **23**, 557–559

www.nature.com/gt

NEWS AND COMMENTARY

In vivo gene therapy potentials of CRISPR-Cas9

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Gene Therapy (2016) **23,** 557–559; doi:10.1038/gt.2016.25;
published online 31 March 2016

Clustered regularly interspaced short palindromic repeats-CRISPR associated nuclease 9 (CRISPR-Cas9) is a genome editing tool derived from microbial adaptive immune defense system.[1] The nuclease Cas9 can generate double-strand breaks (DSBs) on the target DNA sequences in a site-specific way directed by a singly guide RNA (sgRNA) upon the existence of an pro-tospacer adjacent motif (PAM) sequence (Figure 1a).[2–4] The resultant DSDs are repaired by either non-homologous end-joining (NHEJ) or homology-directed repair (HDR), with the former being dominant. NHEJ may give rise to indel mutations, whereas HDR can provide precise gene replacement or addition (Figure 1a). Since its introduction into mammalian cells[2,3] and animals[4] about three years ago, CRISPR-Cas9 has been revolutionizing many fields of medical research and has been applied to the gene therapy explorations of many human diseases.[5–9]

Recently, important progresses have been made in the gene therapy potentials of CRISPR-Cas9. Three studies[10–12] simultaneously published in *Science* reported the ability of CRISPR-Cas9 for *in vivo* gene therapy when delivered locally (intra-muscular) or systemically (intra-peritoneal or intravenous) into adult or neonatal mice with Duchenne muscular dystrophy (DMD), a disease model caused by a nonsense mutation in exon 23 of *Dmd* gene. CRISPR-Cas9 targeting intron 22 and intron 23 of *Dmd* gene can remove the culprit mutation in part of muscle cells and partially restore muscle functions (Figure 1b). In these three studies,[10–12] CRISPR-Cas9 components were delivered through adeno-associated virus vectors (AAV8 or AAV9), which are preferred delivery tools in gene therapy for their broad tissue tropism, low immunogenicity and minimal insertional mutagenicity.[13] To address the package size limitation of AAV vectors, two studies[10–11] used Cas9 from *Staphylococcus aureus* (SaCas9) instead of the commonly used Cas9 from *Streptococcus pyogenes* (SpCas9) because of the smaller size of the former, whereas the third study[12] used spCas9 with a short promoter/enhancer sequence to reduce the package size.

Two other recent studies[14,15] demonstrated the efficacy of CRISPR-Cas9-mediated HDR for *in vivo* gene therapy through intravenous injection. In these two studies, the authors used either a dual viral vector systems[14] or a combination of viral vector and lipid nanoparticles[15] to deliver the three therapeutic components of CRISPR-Cas9 (sgRNA, Cas9 and donor template), and gained a HDR efficiency level sufficient to rescue the disease phenotypes in mouse models of human hereditary liver diseases whose treatments necessitate HDR-mediated gene replacement.

As we know, the application potentials of CRISPR-Cas9 in gene therapy are a fascinating area but the efficacy of *in vivo* delivery is one of the major hurdles. By far, the most majority of therapeutic explorations using CRISPR-Cas9 are conducted in cells or animal germline to rectify, replace or delete the culprit genes.[5,6] However, the strategy of *ex vivo* gene correction followed by autotransplantation or allotransplantation is only applicable to a part of human diseases such as hematological malignancies and may require repeated episodes of treatment, and germline modification is currently unacceptable in humans. In 2014, Yin *et al.*[8] reported *in vivo* gene correction in adult mice with hereditary tyrosinemia by hydrodynamic injection of therapeutic CRISPR-Cas9 components through tail-vein, but this approach remains inapplicable to humans due to its potential damages to liver and cardiovascular functions.[16] On the contrary, these five recent studies[10–12,14,15] demonstrated the effectiveness of CRISPR-Cas9-mediated gene therapy through *in vivo* delivery approaches that are translatable to humans (intra-muscular, intra-peritoneal or intravenous injection). In mouse models of DMD,[10–12] the ratio of dystrophin-positive myofibers was observed to increase with time,[12] indicating that the corrected gene was persistently expressed, and may impose a growth advantage.

In mouse models of human hereditary liver diseases,[14,15] disease phenotypes were rescued by HDR-mediated gene replacement, which has broader applicational spectrum for gene therapy because there are more diseases whose treatment need rectification rather than merely deletion of the culprit genes.

Although these exciting advances imply that we might be not far away from the final applications of CRISPR-Cas9 to human gene therapy, there are still many challenges lying ahead. First, the efficiency of HDR remains to be improved for the gene therapy of diseases whose treatment requires HDR rather than the more efficient NHEJ (Figure 1a). There are several strategies that have been reported to increase HDR efficiency, such as rational design of single-stranded DNA (ssDNA) doners[17] and inhibition of the NHEJ pathway.[18,19] Second, although undetectable or only minimal off-target events were observed in the predicted off-target sites in these three studies,[10–12] they may occur at sites beyond the predicted ones. Therefore, genome-wide unbiased methods such as GUIDE-seq and Digenome-seq should be harnessed to provide a comprehensive profile of the off-target events.[20–22] As off-target genome editing may incur unwanted consequences including malignancies, they should be precisely profiled and reduced to almost nil if applied to human gene therapy. Significant advances have been made recently to reduce the off-target effects, such as double nicking by Cas9 nickases,[23] optimizing sgRNA design[24,25] and reconstruction of Cas9 nuclease.[26] Third, it is still unclear about the functional consequences of the introduction of CRISPR-Cas9 into cells. Studies are needed to investigate how CRISPR-Cas9 components affect cellular functions beyond genome editing and how they alter the endogenous cellular context. Last but not least, the fitness of edited cells can also

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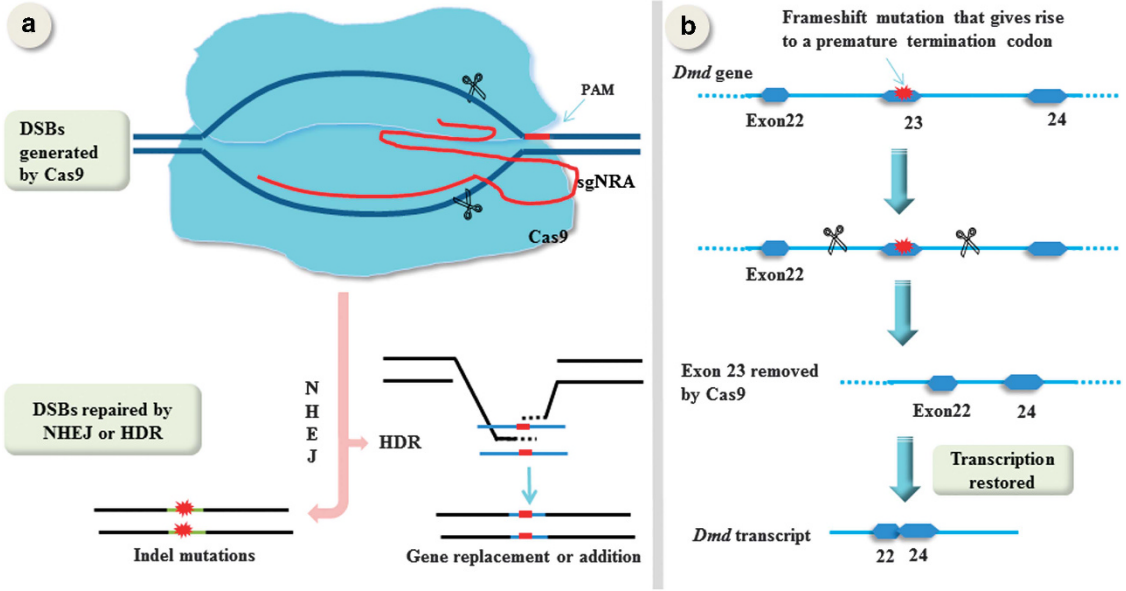

Figure 1. Schematic of CRISPR-Cas9-mediated gene editing and gene correction in mouse with DMD. (**a**) Upon the presence of a pPAM on the opposite strand, the nuclease Cas9 is directed to the target sequence by a sgRNA via base-pairing. Cas9 cuts the target DNA sequence and produces DSBs that are subsequently repaired by either NHEJ or HDR, with the former giving rise to indel mutations, whereas the latter gene replacement or addition. (**b**) The disease-causing mutation for mouse with DMD is a frameshift mutation in the exon 23 of *Dmd* gene that gives rise to a premature termination codon. CRISPR-Cas9-mediated removal of this exon restored *Dmd* transcription and dystrophin protein expression.

influence the effectiveness of CRISPR-Cas9-mediated gene therapy. Persistent efficacy of gene therapy is more likely to be gained for diseases with culprit mutations whose correction confers a growth advantage rather than disadvantage. For example, Yin *et al.*[8] used CRISPR-Cas9 to correct the culprit gene mutation of hereditary tyrosinemia in adult mice, achieving gene corrections in 0.25% of liver cells initially and 33.5% of liver cells 33 days after that was sufficient to rescue the disease phenotype. However, when gene corrections render a growth disadvantage, the genetically corrected cells will be outcompeted by their unedited counterparts. In these cases, CRISPR-Cas9-mediated gene therapy may require pretty high delivery and editing efficiencies and may need repeated episodes of treatment.

In conclusion, these recent advances[10–12,14,15] represent significant steps forward to the final application of CRISPR-Cas9 to human gene therapy. However, there are still many challenges lying ahead, such as delivery efficiency, HDR efficiency, off-target effects and the fitness of edited cells. Future efforts are need to address these challenges to pave the way for the clinical use of CRIPSR-Cas9 as a strategy for gene therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by the National Natural Science Foundation of China (81472242, 81570549) and the Shanghai Municipal Health Bureau Key Disciplines Grant (ZK2015A24).

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