A New Kid on the Playground of CRISPR DMD Therapy

Dongsheng Duan

Departments of Molecular Microbiology and Immunology and Neurology, School of Medicine, Department of Bioengineering, and Department of Biomedical Sciences, College of Veterinary Medicine, The University of Missouri, Columbia, Missouri.

DUCHENNE MUSCULAR DYSTROPHY (DMD) is the most common lethal muscle disease, affecting approximately 250,000 boys worldwide. The disease is caused by mutations in the *dystrophin* gene. Genetic approaches that can repair or replace the mutated gene may radically change the disease course and improve quality of life. Several mechanistically distinctive types of genetic manipulation strategies are currently being explored for treating DMD.^{1,2} These include small molecule read-through of the nonsense stop codon, antisense oligonucleotide-mediated exon skipping of the RNA transcript, adeno-associated virus (AAV)-mediated gene replacement with a <4-kb *microdystrophin* gene and dual-AAV-mediated 6- to 8-kb *minidystrophin* gene therapy, transplantation of heterologous or genetically corrected autologous muscle stem cells, and clustered regularly interspaced short palindromic repeats (CRISPR)-mediated genome editing. Read-through strategy targets the translation step, and it only works for a sub-population of patients. Exon-skipping targets splicing and has to be designed personally for the specific mutation. Both read-through and exon-skipping treatments require repeated administration in order to achieve therapeutic benefits. One read-through drug has been approved in Europe, and one exon-skipping drug has been approved in the United States.^{3–5} Dual AAV minidystrophin therapy has the potential to deliver a genetically optimized minigene that is derived from a naturally existing therapeutic gene in mildly affected Becker muscular dystrophy patients. Success has been achieved in the mouse model of DMD by local and systemic delivery.^{6,7} AAV microgene therapy delivers a synthetic, highly abbreviated gene that encodes a protein about one-third the size

of full-length dystrophin. Systemic microgene therapy has been conducted in the mouse and dog models, and a human trial is slotted for later this year.^{8–11} Preclinical studies suggest that a single intravenous injection of an AAV microgene vector may provide lifelong protection in rodents.

CRISPR therapy is a new type of therapy that has emerged in the last few years.¹² It can remove the mutation from the genome. CRISPR therapy has two major components: an endonuclease called CRISPR-associated protein (Cas) and a guide RNA (gRNA) that directs the Cas to the target site for genome cutting. The Cas protein can be divided into two classes and five types.¹³ Up to now, CRISPR therapy is mainly based on Cas9, a class 2, type II Cas protein. A flurry of papers published in the last 3 years have established the proof of principle for CRISPR DMD therapy using Cas9.^{14–24} Collectively, these studies show effective editing of patient cells *in vitro* and mouse cells *in vivo*. Of high relevance to the development of CRISPR as a therapeutic modality for DMD, several groups delivered the gRNA and Cas9 expression cassette with AAV in mouse models of DMD. Encouragingly, treatment resulted in excellent restoration of dystrophin expression in skeletal muscle and the heart by immunostaining and western blot analysis. Physiological assays also demonstrated improvement of skeletal muscle function.^{15–17,21}

A large collection of Cas endonucleases was discovered in the last couple of years.^{25–28} Many of these newly emerged Cas proteins are capable of genome editing in eukaryotic cells. They represent a rich mine for CRISPR therapy. The unique properties of different Cas proteins offer unlimited opportunities to meet different therapeutic needs.

*Correspondence: Dr. Dongsheng Duan, Department of Molecular Microbiology and Immunology, One Hospital Drive, Columbia, MO 65212. E-mail: duand@missouri.edu

A study published in *Science Advances* on April 12 explored Cpf1, a class 2 type V Cas protein, for DMD therapy in induced pluripotent stem cells derived from a DMD patient and the mdx mouse model for DMD.²⁹ This is the first report demonstrating Cpf1 editing in a mammalian model of a human disease. Cpf1 creates sticky ends in the genome and favors pyrimidine-rich sequences. The Cpf1 gRNA is simpler than that of Cas9. The use of a different protospacer-adjacent motif sequence by Cpf1 expands versatility to genome editing. The authors showed that Cpf1 editing effectively restored the disrupted reading frame and yielded a near-full-length dystrophin protein. In induced pluripotent stem cells–derived cardiomyocytes, Cpf1 editing normalized the mitochondrial number and rescued the respiration rate. Re-implantation of Cpf1-corrected mdx zygotes in pseudopregnant females yielded mutation-corrected mice with normal muscle histology and improved muscle function. Off-target analysis suggests that Cpf1 is highly specific. It is also worth pointing out that in contrast to the published CRISPR strategy, which uses two gRNAs to direct Cas9 to two separate locations in the genome for mutation removal, Zhang *et al.* utilized a very creative approach by directing Cpf1 to cut the splicing acceptor site. This allows restoration of dystrophin expression by one cut, instead of two cuts, and hence may greatly improve the efficiency. Taken together, the results reported by Zhang *et al.* raise a high hope to complement existing Cas9 DMD therapy with Cpf1 (and likely other Cas proteins) for future clinical translation.²⁹

It took about 8 years from the initial description of exon-skipping in mdx myoblasts to the first in-human clinical trial.^{30,31} Similar time was taken

from the report of the first engineered *microdystrophin* gene to the AAV-mediated local injection in DMD boys.^{32,33} It remains to be seen whether CRISPR DMD therapy can follow the same path or may be on the fast track. However, it should be pointed out that there are significant roadblocks ahead of CRISPR therapy, such as off-target toxicity and the immunogenicity of bacteria-derived Cas protein. In the case of CRISPR DMD therapy, there are additional sets of challenges to overcome; for example: How long will therapy last? Will the immune reaction to the Cas protein eventually eliminate the treated dystrophin-positive muscle cells? Will CRISPR therapy improve heart function? Will gene editing effectively target muscle stem cells? Will AAV CRISPR cause any toxicity following long-term *in vivo* expression? Will CRISPR editing work in large mammals? For Cpf1, one still has to show that it can be used as a post-natal therapy for muscle diseases using viral and/or nonviral mediated gene transfer.³⁴

ACKNOWLEDGMENTS

The author thanks Anna Azvolinsky, Rhonda Bassel-Duby, Shi-jie Chen, Chengzu Long, and Eric Olson for helpful discussion. DMD CRISPR therapy in the Duan lab is supported by the National Institutes of Health (AR-69085), Department of Defense (MD150133) and Hope for Javier.

AUTHOR DISCLOSURE

D.D. is a member of the scientific advisory board for Solid Biosciences and an equity holder of Solid Biosciences. The Duan lab has received research support from Solid Biosciences.

REFERENCES

1. Bengtsson NE, Seto JT, Hall JK, et al. Progress and prospects of gene therapy clinical trials for the muscular dystrophies. *Hum Mol Genet* 2016; 25:R9–17.
2. Duan D. Dystrophin gene replacement and gene repair therapy for Duchenne muscular dystrophy in 2016. *Hum Gene Ther Clin Dev* 2016;27:9–18.
3. Muntoni F, Fletcher S, Wilton S. Response to "Railroading at the FDA". *Nat Biotechnol* 2017;35: 207–209.
4. Mendell JR. Eteplirsen improves function and partially restores. *Ann Neurol* 2017;81:164–165.
5. Haas M, Vlcek V, Balabanov P, et al. European Medicines Agency review of ataluren for the treatment of ambulant patients aged 5 years and older with Duchenne muscular dystrophy resulting from a nonsense mutation in the dystrophin gene. *Neuromuscul Disord* 2015;25:5–13.
6. Lai Y, Yue Y, Liu M, et al. Efficient *in vivo* gene expression by trans-splicing adeno-associated viral vectors. *Nat Biotechnol* 2005;23:1435–1439.
7. Zhang Y, Yue Y, Li L, et al. Dual AAV therapy ameliorates exercise-induced muscle injury and functional ischemia in murine models of Duchenne muscular dystrophy. *Hum Mol Genet* 2013;22: 3720–3729.
8. Yue Y, Pan X, Hakim CH, et al. Safe and bodywide muscle transduction in young adult Duchenne muscular dystrophy dogs with adeno-associated virus. *Hum Mol Genet* 2015;24:5880–5890.
9. Bostick B, Shin J-H, Yue Y, et al. AAV-microdystrophin therapy improves cardiac performance in aged female mdx mice. *Mol Ther* 2011;19:1826–1832.
10. Gregorevic P, Allen JM, Minami E, et al. rAAV6-microdystrophin preserves muscle function and extends lifespan in severely dystrophic mice. *Nat Med* 2006;12:787–789.
11. Solid Biosciences. Press Release. <https://solidbio.com/content/stepsforward-duchenne-muscular-dystrophy> (last accessed May 24, 2017).
12. Zhang F. CRISPR-Cas9: prospects and challenges. *Hum Gene Ther* 2015;26:409–410.
13. Makarova KS, Wolf YI, Alkhnbashi OS, et al. An updated evolutionary classification of CRISPR-Cas systems. *Nat Rev Microbiol* 2015;13:722–736.

14. Long C, McAnally JR, Shelton JM, et al. Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. *Science* 2014;345:1184–1188.
15. Long C, Amoasii L, Mireault AA, et al. Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science* 2016;351:400–403.
16. Nelson CE, Hakim CH, Ousterout DG, et al. *In vivo* genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science* 2016;351:403–407.
17. Tabebordbar M, Zhu K, Cheng JK, et al. *In vivo* gene editing in dystrophic mouse muscle and muscle stem cells. *Science* 2016;351:407–411.
18. Ousterout DG, Kabadi AM, Thakore PI, et al. Multiplex CRISPR/Cas9-based genome editing for correction of dystrophin mutations that cause Duchenne muscular dystrophy. *Nat Commun* 2015; 6:6244.
19. Wojtal D, Kemaladewi DU, Malam Z, et al. Spell checking nature: versatility of CRISPR/Cas9 for developing treatments for inherited disorders. *Am J Hum Genet* 2016;98:1–2.
20. Xu L, Park KH, Zhao L, et al. CRISPR-mediated Genome Editing Restores Dystrophin Expression and Function in mdx Mice. *Mol Ther* 2016;24:564–569.
21. Bengtsson NE, Hall JK, Odom GL, et al. Muscle-specific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy. *Nat Commun* 2017; 8:14454.
22. Li HL, Fujimoto N, Sasakawa N, et al. Engineered nuclease mediated genetic correction in iPSCs derived from Duchenne muscular dystrophy patient. *Mol Ther* 2014;22:S124–S124.
23. Young CS, Hicks MR, Ermolova NV, et al. A Single CRISPR-Cas9 deletion strategy that targets the majority of DMD patients restores dystrophin function in hiPSC-derived muscle cells. *Cell Stem Cell* 2016;18:533–540.
24. Maggio I, Stefanucci L, Janssen JM, et al. Selection-free gene repair after adenoviral vector transduction of designer nucleases: rescue of dystrophin synthesis in DMD muscle cell populations. *Nucleic Acids Res* 2016;44:1449–1470.
25. Burstein D, Harrington LB, Strutt SC, et al. New CRISPR-Cas systems from uncultivated microbes. *Nature* 2017;542:237–241.
26. Abudayyeh OO, Gootenberg JS, Konermann S, et al. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science* 2016;353.
27. Shmakov S, Abudayyeh OO, Makarova KS, et al. Discovery and functional characterization of diverse class 2 CRISPR-Cas systems. *Mol Cell* 2015;60:385–397.
28. Zetsche B, Gootenberg JS, Abudayyeh OO, et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 2015;163:759–771.
29. Zhang Y, Long C, Li H, et al. CRISPR-Cpf1 correction of muscular dystrophy mutations in human cardiomyocytes and mice. *Sci Adv* 2017;3: e1602814.
30. Wilton SD, Lloyd F, Carville K, et al. Specific removal of the nonsense mutation from the mdx dystrophin mRNA using antisense oligonucleotides. *Neuromuscul Disord* 1999;9:330–338.
31. van Deutekom JC, Janson AA, Ginjaar IB, et al. Local dystrophin restoration with antisense oligonucleotide PRO051. *N Engl J Med* 2007;357: 2677–2686.
32. Yuasa K, Miyagoe Y, Yamamoto K, et al. Effective restoration of dystrophin-associated proteins *in vivo* by adenovirus-mediated transfer of truncated dystrophin cDNAs. *FEBS Lett* 1998;425:329–336.
33. Mendell JR, Rodino-Klapac LR, Rosales XQ, et al. Sustained alpha-sarcoglycan gene expression after gene transfer in limb-girdle muscular dystrophy, type 2D. *Ann Neurol* 2010; 68:629–638.
34. Zetsche B, Heidenreich M, Mohanraju P et al. Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. *Nat Biotechnol* 2017;35: 31–34.

Received for publication April 28, 2017;
accepted after revision May 8, 2017.

Published online: May 8, 2017.