

Toward the correction of muscular dystrophy by gene editing

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Recent advances in gene editing technologies are enabling the potential correction of devastating monogenic disorders through elimination of underlying genetic mutations. Duchenne muscular dystrophy (DMD) is an especially severe genetic disorder caused by mutations in the gene encoding dystrophin, a membrane-associated protein required for maintenance of muscle structure and function. Patients with DMD succumb to loss of mobility early in life, culminating in premature death from cardiac and respiratory failure. The disease has thus far defied all curative strategies. CRISPR gene editing has provided new opportunities to ameliorate the disease by eliminating DMD mutations and thereby restore dystrophin expression throughout skeletal and cardiac muscle. Proof-of-concept studies in rodents, large mammals, and human cells have validated the potential of this approach, but numerous challenges remain to be addressed, including optimization of gene editing, delivery of gene editing components throughout the musculature, and mitigation of possible immune responses. This paper provides an overview of recent work from our laboratory and others toward the genetic correction of DMD and considers the opportunities and challenges in the path to clinical translation. Lessons learned from these studies will undoubtedly enable further applications of gene editing to numerous other diseases of muscle and other tissues.

myoediting | CRISPR | gene editing

Skeletal and cardiac muscles are essential for all aspects of animal life—from every movement of the body to the second-to-second beat of the heart. There are hundreds of monogenic disorders that disrupt muscle structure and function with devastating clinical consequences (1). Mutations underlying these disorders exist in genes encoding proteins involved in myriad processes, including contractility, membrane integrity, gene regulation, and metabolism. At present, there are no cures for any monogenic muscle diseases and there is a major unmet need and opportunity for the development of new therapeutic strategies to correct the genetic errors that disrupt the expression of essential muscle proteins. The rapid advancement of gene editing technologies with the potential to safely and efficiently modify the genome has transformed this aspiration into a reality (2).

Duchenne Muscular Dystrophy

Among the many monogenic muscle diseases, none is more devastating than Duchenne muscular dystrophy (DMD). First described in 1869 by the French neurologist Guillaume Duchenne, DMD patients lose ambulation at a young age, followed soon thereafter by cardiac and respiratory failure and, ultimately, death, typically by the third decade of life (3, 4). Despite intense effort, DMD has thus far resisted all forms of therapy.

DMD affects ~1:5,000 boys and is the most common fatal monogenic disorder diagnosed in childhood (4). The disease is caused by mutations in the dystrophin gene located on the X chromosome. Two-thirds of DMD cases result from transmission of a mutant dystrophin gene from an unknowing female carrier, whereas one-third of cases are caused by sporadic dystrophin

mutations. The dystrophin gene is the largest gene in the human genome, spanning ~2.3 Mb of DNA (5). The massive dystrophin protein of 427 kDa (>3,600 amino acids) is encoded by 79 exons with splicing patterns that are conserved across vertebrate species, making it possible to extrapolate from studies of dystrophin splicing in mice or other organisms to humans. More than 7,000 different mutations have been identified in DMD patients (6). Most patients contain deletions of one or more exons, but large duplications, complex genomic rearrangements, and point mutations are also frequent. Exon deletions are commonly clustered to specific hot-spot regions between exons 6 and 7 and 43 and 53 (Fig. 1).

Dystrophin maintains the integrity of muscle membranes by linking a cell adhesion complex, referred to as the dystroglycan complex, with the underlying cytoskeleton (7, 8). The N and C termini of dystrophin anchor it to these two structures, respectively, whereas the central region is composed of a series of redundant rod-like domains that function like coils of a spring (Fig. 1). As few as 4 of the 24 rod domains are required for dystrophin function, as revealed in patients with Becker muscular dystrophy (BMD), who have in-frame deletions in this region and display a relatively mild form of the disease (9). Multiple regions of the protein also engage various signaling molecules, such as nitric oxide synthase to modulate muscle function.

Therapeutic Approaches for DMD

Numerous therapeutic approaches have been attempted to restore muscle function in DMD (10, 11). Therapies for DMD should ultimately be directed toward both skeletal and cardiac muscle since both tissues succumb to pathological degeneration. Therapeutic approaches that have been pursued include myoblast and stem cell transfer, up-regulation of the related gene utrophin, treatment with agents that block inflammation, fibrosis, calcium overload and oxidative stress, and even injection with exosomes. Corticosteroid supplementation is current standard of care for DMD patients. However, none of these approaches solve the underlying cause of the disease—the absence of dystrophin.

Extensive efforts have been directed toward the development of oligonucleotides to either mask nonsense codons or skip out-of-frame exons in DMD (12). The first drug approved by the

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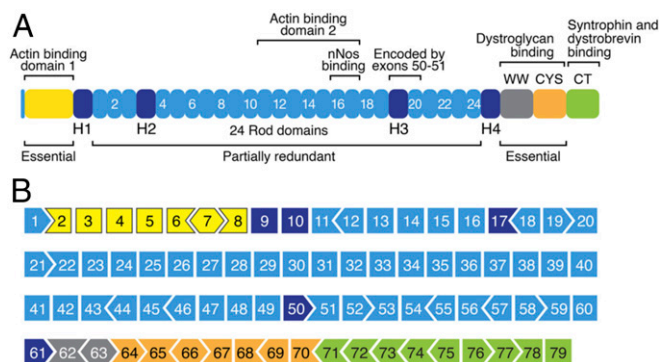


Fig. 1. Dystrophin gene and protein. (A) Schematic of dystrophin protein showing functional domains. Hinge (H), WW, cysteine-rich (CR), and carboxyl-terminal (CT) domains are shown. Myoeediting of a DMD gene lacking exon 50 restores the ORF beginning at rod domain 20. Rod domains are numbered 2 through 24. (B) Splicing of 79 exons of the human dystrophin gene. Colors correspond to functional domains of the protein in A. Shapes of exons indicate whether splicing between adjacent exons maintains the contiguous ORF of the protein when the shapes fit together like pieces of a puzzle. Reproduced with permission from ref. 22. The Annual Review of Medicine © 2019 by Annual Reviews.

Food and Drug Administration as a specific DMD therapy is a morpholino-modified oligonucleotide referred to as eteplirsen (marketed under the trade name Exondys 51), which masks a splice acceptor sequence in exon 51 of the dystrophin gene, thereby promoting exon skipping and restoration of dystrophin expression in patients with deletions of exon 50 (13). After approximately a year of systemic dosing with this drug, muscle biopsies showed ~1% of normal levels of dystrophin protein expression, which is considered to be inadequate to confer significant clinical benefits. Nevertheless, the drug was approved and efforts are underway to develop more effective oligonucleotide therapies. More recently, another oligonucleotide for skipping exon 53 was approved with similar efficacy of ~1% dystrophin restoration after a year of treatment.

Because of the massive size of the dystrophin protein, complete gene replacement strategies are impractical as a means of correcting DMD. However, deletion mutagenesis of dystrophin has identified minimal regions of the protein that are necessary and sufficient for residual function. These proteins, referred to as minidystrophins, which retain the essential regions of the N and C termini of the protein and a minimal number of central rod domains, have been shown to partially substitute for full-length dystrophin in mouse and dog models of DMD (14–16). The design of these truncated forms of dystrophin has also been guided, in part, by the phenotypes of rare BMD patients who harbor large in-frame deletions within the central rod domains but remain ambulatory into the sixth decade of life. Currently, there are several clinical trials underway to express such truncated dystrophins under control of muscle-specific regulatory elements delivered by adeno-associated virus (AAV) (17).

Correction of DMD Mutations by Gene Editing

The advent of gene editing with CRISPR/Cas9 suggested the possibility of correcting mutations responsible for DMD and potentially permanently ameliorating the pathological consequences of the disease. Cas9 and other RNA-guided nucleases are directed to specific DNA sequences through interaction with short guide RNAs (sgRNAs) that anneal to complementary genomic sequences (18–20). When a short protospacer adjacent motif (PAM) is located at an appropriate distance from the sgRNA target sequence, the nucleases generate double-stranded

DNA breaks. Depending on the cell type and the presence or absence of an exogenous DNA template, CRISPR/Cas9 gene editing can occur in either of two ways. Homology-directed repair (HDR), which is confined to proliferating cells, requires an exogenous DNA template and can generate a precise modification at the target locus. Alternatively, in the absence of an exogenous DNA template, Cas9 DNA cutting can result in imprecise DNA repair in which insertions or deletions (INDELs) are created at the site of the double-stranded DNA break, a process referred to as nonhomologous end joining (NHEJ). This form of gene editing can occur in both proliferating and quiescent cells.

There are several aspects of DMD that lend it to CRISPR gene editing as a possible therapeutic approach (21–25). For example, the modular structure of the dystrophin protein with redundancy in the central rod domain permits the deletion of internal segments of the gene that may harbor loss-of-function mutations, thereby restoring the open reading frame (ORF). Such correction strategies would generate forms of dystrophin analogous to those in BMD. The location of the dystrophin gene on the X chromosome also allows for the potential correction of the single mutant allele in affected boys without concerns for inadvertent mutagenesis of a wild-type allele. Moreover, because only a relatively minor fraction of the normal level of dystrophin is required for maintenance of muscle structure and function (26–28), even a modest level of gene correction has the potential to confer significant therapeutic benefits. This contrasts with monogenic disorders in which normal levels of a wild-type protein might be required or in which potentially toxic or dominant negative mutant proteins are produced that require near-complete elimination to achieve therapeutic benefit. Finally, because skeletal muscle is multinucleated and contains hundreds of nuclei in a common cytoplasm, it is possible, in theory, to achieve therapeutic efficacy even if only a fraction of myonuclei are corrected. By one estimate, correction of only 4% of myonuclei could be sufficient to restore dystrophin protein expression along the lengths of myofibers, at least in mice (29).

In initial proof-of-concept studies, we used the mdx mouse model of DMD, which harbors a dystrophin nonsense mutation in exon 23, and tested whether injection of *Streptococcus pyogenes* Cas9 (SpCas9), sgRNA, and a DNA template could correct the nonsense mutation by HDR and restore dystrophin expression (30). These studies were performed in injected mouse zygotes, followed by reimplantation into pseudopregnant females. Offspring from these mice were mosaic because Cas9-mediated HDR does not occur until after the zygote stage, resulting in genomic editing in a subset of embryonic cells. This mosaicism allowed us to determine the minimum level of gene editing required to restore dystrophin protein expression throughout skeletal muscle. From these studies, we obtained genetically mosaic animals containing 2 to 100% correction of the dystrophin gene and found that only ~15% of gene editing allowed the expression of dystrophin at nearly wild-type levels throughout skeletal muscle and heart and rescued pathological aspects of the disease. Similar results were obtained from germline editing of mdx mice with Cpf1, a unique class 2 CRISPR effector with a different PAM sequence than SpCas9 (31). However, because germline editing is not currently feasible or ethical in humans, we and others have attempted to further optimize the method for correction of postnatal muscle cells in vitro and in vivo.

There are several significant challenges for DMD correction by gene editing. For example, HDR cannot be deployed as a mechanism for precise DMD correction because muscle cells are permanently postmitotic and nonpermissive to this process. Second, considering the thousands of known DMD mutations, it is impractical to develop and optimize a gene correction strategy unique to each individual mutation. Thus, it is necessary to consolidate numerous mutations for correction by a shared strategy and to perform the simplest possible modification of the genome

so as to minimize possible deleterious or unpredictable mutations. Finally, gene editing components must be delivered efficiently *in vivo* to the entire skeletal musculature and the heart.

These challenges can be addressed by a gene editing strategy in which Cas9 is directed to introduce a single double-stranded break in the DNA of out-of-frame dystrophin exons, followed by DNA repair through NHEJ and reconstitution of the ORF (29, 32–36). We refer to this method as single-cut myoeediting, because only one sgRNA is used to direct Cas9 editing in targeted muscle cells. With single-cut myoeediting, the DMD reading frame can potentially be corrected in either of two ways: 1) An out-of-frame exon can be skipped through deletion of a splice donor or acceptor or 2) the dystrophin protein can be reframed by deletion or insertion of the appropriate number of nucleotides to restore the correct alignment of triplet codons (Fig. 2). As discussed below, single-cut myoeediting allows the efficient and safe correction of greater than 60% of DMD mutations with a relatively small number of optimized sgRNAs. An alternative strategy for the correction of DMD mutations employs Cas9 with a pair of sgRNAs to introduce two double-stranded DNA breaks flanking large genomic hot-spot regions (37–39). While this approach can, in principle, be applied to a large percentage of DMD mutations, there are at least two limitations to this approach. Double-cut gene editing requires the introduction of simultaneous double-stranded DNA breaks separated by large genomic distances, which is highly inefficient. For example, to remove mutational hot-spot regions in the DMD gene requires deletion of a genomic fragment of up to 725 kb. In addition, double cutting of such a large DNA region is associated with complex and unpredictable genomic rearrangements that pose significant safety issues. Moreover, the requirement for two different sgRNAs raises an additional challenge for delivery, since sgRNAs are limiting for the efficacy of gene editing (40–42).

Using cardiomyocytes derived from human induced pluripotent stem cells (iPSCs), we showed that dystrophin expression could be restored following single-cut gene editing of diverse types of DMD mutations (35, 36, 40). Gene correction in iPSC-derived cardiomyocytes can be especially informative not only for demonstrating efficacy of gene editing with individual sgRNAs but also for testing whether the form of the dystrophin protein generated by a particular editing strategy is stable, localized appropriately to the sarcolemma, and capable of supporting cardiomyocyte contractility. Moreover, reconstitution of edited cardiomyocytes into three-dimensional human engineered

heart muscle tissue *in vitro* allows for measurements of contractile force of corrected cardiomyocytes (36). Such studies can also predict possible clinical outcomes of various gene editing events, since the functionality and stability of different dystrophin deletion mutants cannot always be predicted *a priori*. This was exemplified for gene editing of a DMD deletion mutation in the actin-binding region of the gene, such that three different multiexon deletion strategies to restore the ORF resulted in forms of the protein with very different functions and stabilities (39).

Dystrophin Restoration and Correction of DMD *In Vivo*

The most common DMD mutational hotspot involves the deletion of exon 50, which places exon 51 out-of-frame with preceding exons. An example of the single-cut myoeediting strategy for correction of an exon 50 deletion is shown in Fig. 2. In an effort to restore dystrophin expression in DMD, we reasoned that directing Cas9 to an out-of-frame exon using a guide proximal to the splice acceptor site of the exon might allow either exon skipping or reframing depending on the size and nature of INDELs introduced at the double-stranded DNA break.

To test the therapeutic potential of single-cut myoeediting *in vivo*, we have generated mice harboring each of the most commonly deleted exons in DMD patients (35, 40, 41). To deliver SpCas9 and sgRNAs *in vivo*, we and others have used AAVs of serotype 9 (AAV9) (34, 35, 37, 40–51). The SpCas9 cDNA is at the size limit that can be packaged into AAV9, necessitating the delivery of sgRNAs in a separate AAV9 cassette. *Staphylococcus aureus* Cas9 (SaCas9) is smaller than SpCas9 and has also been used for DMD gene editing (50, 51). However, the PAM sequence for SaCas9 is more complex than that of SpCas9, which limits the potential editing events that can be introduced. We used the muscle creatine kinase (MCK) promoter to direct the muscle-specific expression of Cas9 (52) and three different RNA polymerase promoters (U6, H1, and 7SK) to direct the expression of sgRNAs from a separate AAV9 cassette (53). We selected the MCK promoter for our initial studies because of its high-level expression in terminally differentiated skeletal and cardiac muscle and because of its inactivity in the liver, which is a site of AAV9 accumulation *in vivo*. The inactivity of the MCK promoter in liver minimizes possible off-target gene editing in this tissue. Systemic dosing of mice harboring an exon 50 deletion with AAV9-Cas9 and AAV9 encoding an sgRNA directed against a sequence in exon 51 allowed efficient restoration of dystrophin protein expression within 4 wk, as well as maintenance of muscle strength and function and prevented pathological myofiber degeneration and fibrosis (35).

Analysis of gene editing at the target site by genomic and cDNA amplicon deep sequencing revealed an optimal sgRNA that allowed the insertion of a single adenosine nucleotide at the double-stranded DNA break. This type of single nucleotide insertion occurs due to the creation of a one-nucleotide overhang at the site of DNA cleavage, which is filled by DNA polymerase and ligated to the adjacent DNA end (35). For exons that are out of frame by a single nucleotide, this form of editing can restore the ORF of the protein by inserting one nucleotide (+3n + 1 insertion) with the minimal possible modification of the genome. For example, when we used one sgRNA to direct Cas9 cleavage by single-cut gene editing of exon 45 in cultured cells and mice lacking exon 44, which disrupted the dystrophin ORF, we observed efficient insertion of one nucleotide at the DNA cutting site, which restored dystrophin protein expression (40).

To determine whether myoeediting might be applicable to larger animals, we tested the approach in a dog model of DMD that harbors a missense mutation in the 5' splice site of exon 50 that results in deletion of exon 50 in dystrophin transcripts and premature termination of dystrophin translation due to a nonsense codon when exon 49 splices to exon 51 (54). This model closely resembles human DMD with respect to muscle pathology,

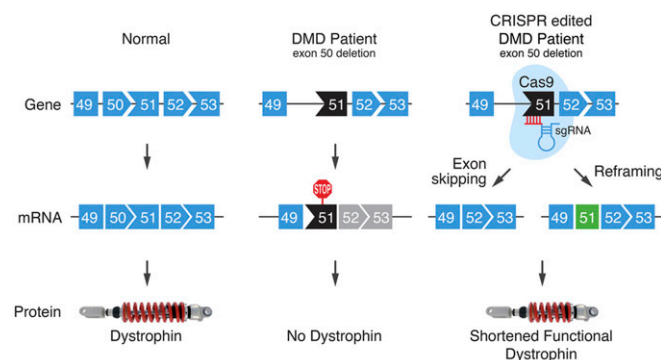


Fig. 2. Single-cut gene editing of an exon 50 DMD deletion. Normal DMD gene and mRNA from exon 49 to 53 is shown (Left). An exon 50 DMD mutation is shown (Middle). Splicing of exon 49 to exon 51 places the dystrophin protein out of frame due to a stop codon. Single-cut gene editing (Right) with an sgRNA directed against a sequence within exon 51 can restore dystrophin expression by exon skipping or reframing, depending on the type of INDEL introduced. Dystrophin protein is schematized as a shock absorber.

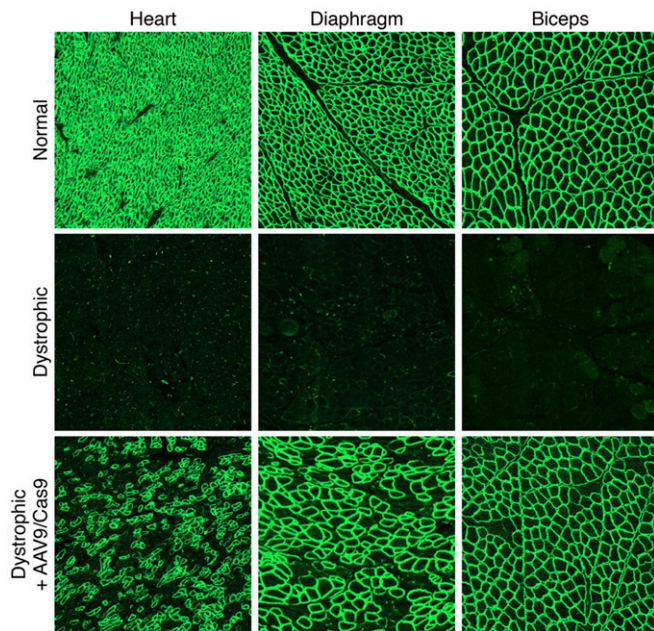


Fig. 3. Dystrophin immunostaining in muscle tissues of a canine model of DMD. (Upper) Dystrophin in muscle tissues from wild type. (Middle) Absence of dystrophin in Δ Ex50 dog. (Lower) Restoration of dystrophin expression in Δ Ex50 dog 8 wk after AAV9 delivery of gene editing components. Adapted from ref. 55, with permission from AAAS.

atrophy, fibrosis, and loss of ambulation at a young age. AAV9-Cas9 and AAV9-sgRNA-51 expression cassettes were delivered by intravenous infusion at 1 mo of age at two doses of 2×10^{13} viral genomes (vg)/kg and 1×10^{14} vg/kg each and dogs were analyzed 8 wk later (55). To avoid a possible immune reaction, we included a transient regimen of immunosuppression with prednisolone with the higher dose. Both doses of AAV9 vectors resulted in pronounced restoration of dystrophin expression throughout all muscles, including the diaphragm and the heart, with higher dystrophin expression at the highest dose (Fig. 3). Western blotting showed between 3 and 90% of normal dystrophin levels in different skeletal muscles and 92% of normal in the heart with the highest dose. Amplicon deep-sequencing analysis of genomic DNA from the heart and different skeletal muscles showed preferential insertion of a single adenosine, along with a variety of other small insertions and deletions, at the target site in exon 51, just as seen in mice and human cells. It should be pointed out that the DNA sequencing method we deployed would not detect large deletions, but these appear to represent a relatively minor fraction of INDELs and would not be expected to diminish the benefits of smaller INDELs that restore the dystrophin ORF. As expected from the muscle specificity of the MCK promoter used to drive expression of Cas9, gene editing was confined to muscle tissues and was not observed in testes. No evidence for abnormalities in hematology counts, CD4- or CD8-positive cell infiltration of treated muscle, serum electrolytes, and kidney or liver function were observed in any treated dogs, nor was off-target mutagenesis observed based on deep-sequencing analysis of top predicted off-target sites.

Recently, systemic delivery of AAV9 expressing SpCas9 using a split intein system and a pair of sgRNAs flanking exon 51 in a pig model of DMD lacking exon 52 was also shown to restore dystrophin expression and to prevent muscle pathology and cardiac arrhythmias associated with the disease within 3 to 4 mo (56). Coating of AAV9 with two polyamidoamine nanoparticles

was employed to increase tropism of the vector to muscle without toxicity.

Noninvasive Monitoring of Gene Editing in Mice

A challenge with respect to monitoring DMD gene correction strategies *in vivo* is the necessity to isolate and analyze tissues during the course of the disease. To circumvent this challenge, we generated dystrophin reporter mice in which a luciferase expression cassette is introduced into the genome immediately 3' of the dystrophin coding region such that luciferase is expressed as a fusion protein with dystrophin (57). Separation of the two coding regions with an autocatalytic protease 2A cleavage site allows for the release of luciferase from dystrophin and detection of luciferase non-invasively as a proxy for dystrophin expression. Through introduction of DMD exon deletions in these mice, followed by AAV delivery of myoediting components, we monitored the restoration of luciferase expression and found that myoediting and dystrophin expression are readily detectable within 1 wk of injection and rapidly achieve maximal and sustained levels by 1 mo.

Gene Editing versus Microdystrophin Gene Therapy and Oligonucleotide Delivery

There are several key distinctions between gene editing and microdystrophin gene replacement as potential therapies for DMD. Single-cut gene editing requires specific sgRNAs that vary depending on the deletion mutation being corrected, whereas microdystrophin gene replacement can be deployed against virtually any DMD mutation. In addition, while single-cut gene editing has the potential to restore dystrophin expression in up to 60 to 80% of DMD mutations, there are also mutations that cannot be corrected by this approach. These include large deletions that encompass essential portions of the N- or C-terminal domain of the protein. Another key distinction between gene editing and microdystrophin gene replacement is the differences in dystrophin regulation between the two approaches. For single-cut gene editing, the corrected dystrophin gene is expressed from the endogenous locus and is therefore subject to normal regulation with respect to tissue specificity, timing, and levels of expression, as opposed to gene replacement approaches that rely on the regulatory elements in the vector. CRISPR-edited DMD alleles may also be expected to be expressed for a sustained period of time, perhaps permanently. DMD correction by gene editing also allows for expression of the largest possible form of dystrophin, whereas gene replacement is restricted to truncated proteins containing only ~30% of the full-length protein and are therefore highly limited in function. For example, in the case of reframing of exon 51 for correction of an exon 50 deletion by single-cut gene editing, only the short hinge 3 region of dystrophin is missing following myoediting.

There are also key differences between gene editing and oligonucleotide therapies for exon skipping that are worth mentioning. Whereas CRISPR/Cas9 editing may be long-lasting and not require redelivery, oligonucleotide therapies require life-long continuous treatment and, thus far, have not shown significant efficacy with respect to dystrophin restoration, despite being safe. However, it should also be mentioned that CRISPR treatment may be difficult to terminate if safety issues are encountered.

Future Questions and Potential Challenges

Despite promising results for the correction of DMD mutations in mice, dogs, and pigs following AAV9 delivery of gene editing components, there are several unknowns that remain to be fully addressed. For example, how long might the benefits of gene editing be sustained in DMD? Sustained genome editing and

dystrophin expression for 12 to 18 mo has been reported in mdx mice after AAV delivery of gene-editing components (42, 44). We have also observed the maintenance of dystrophin expression in skeletal muscle for over a year following single-cut gene editing. In this regard, skeletal muscle is a long-lived tissue but the potential rate in which myoedited nuclei might be lost over time remains to be determined. Skeletal muscle is also among the most regenerative of adult tissues, owing to the existence of an intrinsic population of stem cells, known as satellite cells, that reside beneath the basal lamina of mature muscle fibers. In response to injury or aging, satellite cells become activated and fuse with residual myofibers to maintain muscle mass and function. In chronic degenerative muscle disorders, the satellite cell population becomes depleted, exacerbating muscle dysfunction. AAV9 is generally not thought to efficiently infect satellite cells, although there have been reports of low-level gene editing in satellite cells of mice following AAV9 delivery of gene editing components (45, 48, 58). Even without satellite cell gene editing, fusion of satellite cells to regenerating myofibers would likely expose satellite cell nuclei to gene editing machinery in the myofiber and might thereby enable those nascent nuclei to express dystrophin. In contrast to skeletal muscle, which displays some turnover, cardiomyocytes are permanent, so we would anticipate that gene correction in those cells could be life-long.

Off-target mutagenesis is a frequently cited concern for gene editing (59, 60). In our studies thus far, we have observed only very low levels of off-target editing (less than 1%), but this will need to be further assessed in the future. Most studies in which off-target editing was reported involved highly proliferative cells in culture with high doses of gene-editing components. However, because muscle cells are permanently postmitotic and tumors do not form from terminally differentiated muscle cells, off-target effects may be less of a concern for a disease such as DMD. Moreover, the levels of expression of gene-editing components that are achieved in vivo are far lower than in cultured cells, reducing the likelihood of extensive off-target mutagenesis. High-fidelity forms of Cas9 with diminished off-target mutagenesis may also provide further safety (61–63). A concern for all AAV-dependent gene therapies is possible integration of the AAV vector into the genome (51, 64, 65). Analysis of such insertions in mice following systemic delivery of AAV9-Cas9 and sgRNAs revealed very low level of insertion of a short segment of the inverted terminal repeat region of AAV at the site of CRISPR DNA cutting, but this did not appear to have pathological consequences (40).

Manufacturing of clinical-grade AAV in the high quantities required for treatment of large numbers of patients also remains a significant challenge for all types of gene therapies (66). Current dosing regimens in animal models as well as in patients require doses in the range of 1×10^{14} vg/kg for effective expression in muscle tissue. There is a major need for new strategies to lower these doses by optimizing AAV production, infectivity, tissue-specific tropism, and expression. Regarding liver toxicity, recent studies in primates as well as initial human trials with AAV as a delivery vehicle have observed acute liver toxicity at high doses, which has been attributed at least in part to activation of the innate immune response (16).

CRISPR/Cas9 myoediting in its current iterations requires two viral vectors—one to direct the expression of SpCas9 and the other to direct expression of sgRNAs. Titration of the two vectors in vivo has shown that sgRNAs are limiting for the efficiency of gene editing in skeletal muscle and a ratio of sgRNA:Cas9 AAVs of up to 10:1 can dramatically enhance the efficiency of gene correction in skeletal muscle (40, 42, 43). In contrast, the heart is more efficiently transduced by AAV and can achieve maximal gene editing at doses of AAV at least 10 times lower than in skeletal muscle and without the marked dependency on sgRNA dose (40, 43).

Recently, we reported that self-complementary AAV (scAAV) in which the viral DNA is packaged in a double-stranded form is significantly more efficient in directing myoediting than single-stranded AAV (43). Unlike the conventional single-stranded AAV, scAAV vectors contain a mutation that allows bypass of second-strand synthesis, which is a rate-limiting step for gene expression (66). Double-stranded scAAV is also resistant to DNA degradation after viral transduction, thereby allowing higher copy number of stable episomes. Because the size limitation for self-complementary AAV is ~2.1 kb, which exceeds the size of the Cas9 coding region, only the sgRNAs can be delivered in this form. There is a need for further optimization of vector design and, if possible, the incorporation of sgRNA and Cas9 cassettes into a single AAV vector. Off-switches or the use of unstable forms of Cas9 would also be beneficial as a means of diminishing possible adverse effects of Cas9 over the long term.

While AAV has provided the most effective means of systemic delivery of gene editing components to date, gene delivery using nanoparticles has also been demonstrated in cultured cells and at sites of intramuscular injection in vivo and has been reported to enable modest restoration of dystrophin expression in mouse models of DMD mice (67, 68). However, this approach will need to overcome challenges of systemic in vivo delivery to muscles and the heart.

There are several important issues of immunity that warrant careful consideration. AAV-neutralizing antibodies are common in the human population and anti-SpCas9 antibodies and SpCas9-specific T cells have also been reported in humans (69–74). Thus, a subset of potential patients will be ineligible for AAV therapy due to prior exposure and those patients who are treated can only receive a single dose. If additional AAV injections are required, perhaps alternative AAV serotypes might mitigate such responses (75). In addition, infusion of the immunoglobulin G-cleaving endopeptidase imlifidase was recently shown to reduce anti-AAV antibody levels in mice, thereby allowing enhanced AAV transduction in the setting of vector readministration (76). As a bacterial protein, Cas9 will likely trigger an immune response, as has been reported by others (72). Thus far, we have not observed a significant immune response in mice or dogs following systemic delivery of AAV9-Cas9, but further studies are needed. The possible use of modified forms of Cas9 lacking immunodominant epitopes has also been described (77). It is noteworthy in this regard that CRISPR-based gene silencing has been reported to elicit a Cas9-dependent host response that resolved without intervention (51). In addition, DMD patients are typically maintained on corticosteroids to reduce inflammation, which might dampen possible immune responses. Finally, dystrophin itself could trigger an immune response in a patient. However, this may be less of a concern because DMD patients have “revertant fibers,” representing up to 1% of total myofibers, that express dystrophin due to imprecision of splicing in which a small fraction of transcripts may contain the dystrophin ORF, thereby allowing dystrophin to be recognized as “self,” and avoiding an immune response after therapy.

What would be the optimal age to intervene with myoediting in a DMD patient, and when is too late? It is our view that the earlier the intervention the better for several reasons. First, the younger the patient, the more muscle is preserved, whereas in late stages of the disease muscle is replaced by fibrous tissue and fat that do not have muscle-building potential. In this regard, our results to date in animal models indicate that myoediting can preserve muscle from the time of intervention but cannot create muscle de novo. From a practical point of view, the younger the patient, the lower the dose of AAV that would need to be delivered, thereby minimizing possible liver toxicity. Finally, younger patients are less likely to have preexisting immunity against AAV9.

Looking to the Future

DMD has defied all therapies since its initial description 150 y ago. CRISPR gene editing represents the latest in a long line of attempted therapies but is distinguished from others by its potential to eliminate the cause of the disease rather than simply treating the symptoms. While this paper has focused on correction of DMD mutations by CRISPR/Cas9, numerous other gene editing strategies are being developed, including precise editing of single nucleotides by base editing, which allows the conversion of a single target base or base pair into another (e.g., A:T to G:C or C:G to T:A) without requiring the creation of double-stranded DNA breaks in the target DNA (78). Ultimately,

the lessons learned from DMD should be applicable not only to other muscle diseases but also to many other devastating genetic disorders for which there are no effective therapies.

Data Availability. There are no data underlying this work.

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