this approach to confer body-wide therapeutic benefits. The accompanying articles from Wagers and colleagues (41) and Olson and colleagues (42) adopt a similar approach to CRISPR-Cas9-based correction of dystrophic mice using delivery with AAV9, demonstrating generality across muscle-tropic AAV serotypes. Moreover, the Wagers group’s demonstration of efficient editing of Pax7-positive muscle satellite cells (41) suggests that gene correction may improve as the mature muscle fibers are populated with the progeny of these progenitor cells, as was observed in a muscular dystrophy mouse generated by a CRISPR-Cas9 delivery to single-cell zygotes (27). Indeed, we have observed that dystrophin restoration by genome editing is maintained for at least 6 months after treatment (Fig. S14).

Continued optimization of vector design will be important for potential clinical translation of this approach, including evaluation of various AAV capsids and tissue-specific promoters. Additionally, although dual-vector administration has been effective in body-wide correction of animal models of DMD (43), optimization to engineer a single-vector approach may increase efficacy and translatability. These three studies (41, 42) establish a strategy for gene correction by a single-gene editing treatment that has the potential to achieve effects similar to those seen with weekly administration of exon-skipping therapies (8, 9, 30, 31). More broadly, this work establishes CRISPR-Cas9-mediated genome editing as an effective tool for gene modification in skel- etal and cardiac muscle and as a therapeutic approach to correct protein deficiencies in neuromuscular disorders and potentially many other diseases. The continued development of this technology to characterize and enhance the safety and efficacy of gene editing will help to realize its promise for treating genetic disease.

REFERENCES AND NOTES


GENE EDITING

In vivo gene editing in dystrophic mouse muscle and muscle stem cells

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Frame-disrupting mutations in the DMD gene, encoding dystrophin, compromise myofiber integrity and drive muscle deterioration in Duchenne muscular dystrophy (DMD). Removing one or more from the mutated transcript can produce an in-frame mRNA and a truncated, but still functional, protein. In this study, we developed and tested a direct gene-editing approach to induce exon deletion and recover dystrophin expression in the mdx mouse model of DMD. Delivery by adeno-associated virus (AAV) of clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 endonucleases coupled with paired guide RNAs flanking the mutated Dmd exon23 resulted in excision of intervening DNA and restored the Dmd reading frame in myofibers, cardiomyocytes, and muscle stem cells after local or systemic delivery. AAV-Dmd CRISPR treatment partially recovered muscle functional deficiencies and generated a pool of endogenously corrected myogenic precursors in mdx mouse muscle.

Duchenne muscular dystrophy (DMD) is a progressive muscle degenerative disease caused by point mutations, deletions, or duplications in the DMD gene that cause genetic frame-shift or loss of protein expression (1). Efforts under development to reverse the pathological consequences of DYSTROPHIN deficiency in DMD aim to restore its biological function through viral-mediated delivery of genes encoding shortened forms of the protein, up-regulation of compensatory proteins, or inter-

REFERENCES (see www.sciencemag.org/content/351/6271/403/suppl/DC1 SUPPLEMENTARY MATERIALS www.sciencemag.org/content/351/6271/403/suppl/DC1 Materials and Methods Figs. S1 to S22 Tables S1 to S4 References (44–48) 24 September 2015; accepted 7 December 2015 Published online 31 December 2015 10.1126/science.aaf5143


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mRNA, to restore biologically active DYSTROPHIN protein in mice (5, 6) and humans (7, 8). Yet limitations remain for the use of AONs, including variable efficiencies of tissue uptake, depending on antisense oligonucleotide (AON) chemistry, a requirement for repeated AON injection to maintain effective skipping, and the potential for AON-associated toxicities (9, 10) and supplementary text.

Here, we sought to address these limitations by developing a one-time, multisystemic approach based on the genome-editing capabilities of the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system. This system, originally coopted from Streptococcus pyogenes (Sp), couples a DNA double-strand endonuclease with short “guide” RNAs (gRNAs) that provide target specificity to any site in the genome that also contains an adjacent “NGG” protospacer-adjacent motif (PAM) (11–14), which enables targeted gene disruption, replacement, and modification.

To apply CRISPR/Cas9 for exon deletion in DMD, we first established a reporter system for CRISPR activity by “repurposing” the existing A9 mouse reporter allele, which encodes the fluorescent tdTomato protein downstream of a ubiquitous CAGGS promoter and “fooled” STOP cassette (15, 16) (Fig. S1A). Exposure to SpCas9, together with paired gRNAs targeting near the A9 loxP sites (hereafter, A9 gRNAs), resulted in excision of intervening DNA and expression of tdTomato (fig. S1, A, B, and E). We next designed and tested paired gRNAs (hereafter, Dmd23 gRNAs) (fig. S1C) that were directed 5′ and 3′ of mouse Dmd exon 23, which in mdx cells carries a nonsense mutation that destabilizes Dmd mRNA and disrupts DYSTROPHIN expression (17). Finally, we coupled the paired Dmd23 and A9 gRNAs using a two-plasmid system that links expression of the CRISPR activity reporter (tdTomato) to genome editing events at the Dmd locus (fig. S1D).

In vitro transfection of primary satellite cells from mdx mice carrying the A9 allele (hereafter, mdx:A9 mice) with SpCas9 + A9-Dmd23 coupled gRNAs induced gene editing at both the A9 locus, demonstrated by tdTomato expression (fig. S1E), and Dmd locus, detected by genomic polymerase chain reaction (PCR) (Fig. 1A) and confirmed by amplicon sequencing (fig. S1F). Dmd editing was not detected in mdx:A9 cells receiving A9 gRNAs alone (Fig. 1A), although tdTomato expression was equivalently induced (fig. S1E).

To confirm that CRISPR-mediated Dmd editing results in irreversible genomic modification and production of exone-deleted mRNA and protein, primary satellite cells from mdx:A9 mice were cotransfected with SpCas9 + A9 or A9-Dmd23 gRNAs, isolated by fluorescence-activated cell sorting (FACS) on the basis of tdTomato expression, expanded in vitro (18), and differentiated to myotubes. Reverse transcription–PCR (RT-PCR) (Fig. 1B) and amplicon sequencing (fig. S1G) from these myotubes detected exon 23–deleted Dmd mRNA in cells receiving A9-Dmd23–coupled gRNAs but not in cells receiving only A9 gRNAs. TaqMan analysis (9) further indicated that exon 23–deleted transcripts represented 24 to 47% of total Dmd mRNA in cells receiving A9-Dmd23–coupled gRNAs, whereas exon 23 deletion was undetectable with A9 gRNAs alone (fig. S1H). DYSTROPHIN protein expression was also restored in CRISPR-modified mdx:A9 cells, as detected by Western blot of in vitro differentiated myotubes (Fig. 1C) and immunostaining of muscle sections from mdx mice transplanted with gene-edited mdx:A9 satellite cells (Fig. 1D and fig. S1I). These data demonstrate that CRISPR/Cas9 can direct sequence-specific modification of disease alleles in primary muscle stem cells that retain muscle engraftment capacity.

We next adapted CRISPR for delivery by means of AAV, using the smaller Cas9 ortholog from Staphylococcus aureus (SaCas9), which can be packaged in AAV and programmed to target any locus in the genome containing an “NGRRT” PAM sequence (19). We generated Sa gRNAs targeting A9 and introduced several base modifications into the gRNA scaffold to enhance gene targeting by SaCas9 (fig. S2, A to C). Using this modified scaffold, we tested Dmd23 Sa gRNAs (fig. S2D) and produced AAVs encoding SaCas9 and A9 Sa gRNAs or Dmd23 Sa gRNAs in a dual (fig. S3A) or single (fig. S3B) vector system. Comparison of exon 23 excision efficiencies in transduced mdx myotubes demonstrated more efficient excision by dual AAV-CRISPR (fig. S3, C and D), as compared with single vector AAVs. Therefore, to test the potential for in vivo Dmd targeting by CRISPR/Cas9, we pseudotyped dual AAVs (AAV-SaCas9 + AAV-A9 gRNAs; hereafter, AAV-A9 CRISPR) to serotype 9, which exhibits robust transduction of mouse skeletal and cardiac muscle (20), and injected these AAVs into the tibialis anterior (TA) muscles of mdx:A9 mice (7.5E+11 vg each). Four weeks later, muscles were harvested to assess genome-editing events. TdTomato fluorescence was detected in muscles injected with AAV-A9 CRISPR but not in muscles injected with vehicle alone (fig. S4A), Codelivery of AAV9-SaCas9 + AAV9-Dmd23 gRNAs (hereafter,
AAV-Dmd CRISPR likewise yielded robust and specific modification of the Dmd locus in TA muscles in vivo. Genomic PCR (Fig. 2A) and Sanger sequencing (fig. S4B) demonstrated exon 23 excision in muscles injected with AAV-Dmd CRISPR but not AAV-Ai9 CRISPR. Next-generation sequencing indicated minimal activity at the predicted highest-ranking genomic off-target sites (fig. S12). RT-PCR (Fig. 2B) and sequencing (fig. S4C) further confirmed the presence of exon 23-deleted Dmd mRNA in muscles receiving AAV-Dmd CRISPR, with an average exon 23 excision rate of 39% ± 18% (fig. S12E). In vivo CRISPR-mediated targeting of Dmd exon 23-restored DYSTROPHIN expression in skeletal muscle, as detected by Western blot (Fig. 2C), immunofluorescence (Fig. 2D), and capillary immunoassay (fig. S5A). Other pathological hallmarks of dystrophy were also restored in AAV-Dmd CRISPR-injected muscles, including sarcolemmal localization of the multimeric dystrophin-glycoprotein complex and neuronal nitric-oxide synthase (figs. S6 and S7). In contrast, DYSTROPHIN expression was undetectable by Western blot (Fig. 2C) and present only on rare revertant fibers in mdx;Ai9 mice receiving control AAV-Ai9 CRISPR (Fig. 2D) (2D). Finally, to evaluate the functional consequences of CRISPR-mediated induction of exon 23-deleted DYSTROPHIN, we subjected a subset of mdx;Ai9 mice injected intramuscularly with AAV-Dmd CRISPR to in situ muscle force assessment. Muscles receiving AAV-Dmd CRISPR showed significantly increased specific force (Fig. 2E) and attenuated force drop after eccentric damage (Fig. 2F), as compared with contralateral, vehicle-injected muscles and also AAV-Ai9 CRISPR injected muscles. In contrast, differences in specific force (Fig. 2E) and force drop (Fig. 2F) for AAV-Ai9 CRISPR injected mice did not vary significantly between the virus-injected and vehicle-injected muscles.

We next evaluated the potential for multisystemic gene editing in vivo using AAV-CRISPR. Dual AAV-Ai9 CRISPR vectors (1.5E+12 vg each) were coinjected intraperitoneally into mdx;Ai9 mice at postnatal day 3 (P3). Three weeks later, widespread tdTomato expression was detected in all cardiac and skeletal muscles and cardiac muscle, with targeting levels varying from 3 to 18% in different muscle groups (Fig. 3A and fig. S3F). Exon 23 was not excised in animals receiving AAV-Ai9 CRISPR instead (Fig. 3A, and figs. S3F and S8B). Finally, Western blot (Fig. 3B) and fig. S8C), immunofluorescence (Fig. 3C), and capillary immunoassay (fig. S5B) confirmed that DYSTROPHIN protein was largely absent in muscles of control mdx;Ai9 mice receiving AAV-Ai9 CRISPR and was restored in mice receiving AAV-Dmd CRISPR. Similar systemic dissemination of AAV and excision of exon 23 in multiple organs were seen in two adult mice injected intravenously with AAV-Dmd CRISPR at 6 weeks of age (fig. S9).
Dystrophic pathology and other muscle injuries activate muscle stem cells (also known as satellite cells), which leads to regenerative responses that add new nuclei to damaged fibers (2) and supplementary text. To evaluate AAV-CRISPR gene editing in satellite cells in vivo, we crossed mdx;Ai9 mice with Pax7-ZsGreen animals, in which satellite cells are specifically marked by green fluorescence (22), and we injected these animals intramuscularly or systemically with AAV9 encoding Cre (hereafter, AAV-Cre) or Ai9-CRISPR components. Muscles were harvested 2 weeks later (Fig. 4A) and analyzed by FACS. TdTomato expression was apparent in Pax7-ZsGreen+ satellite cells after intramuscular or systemic delivery of AAV-Dmd CRISPR or AAV-Ai9 CRISPR. Satellite cells were isolated by FACS, expanded, and differentiated in vitro (Fig. 4A). RT-PCR revealed a truncated Dmd transcript of the expected size and sequence for gene-edited Dmd in cell-derived myotubes from many of the AAV-Dmd CRISPR-injected muscles but none of the AAV-Ai9 CRISPR-injected muscles (Fig. 4D and fig. S10, F, H, and I). Quantification of exon 23 excision revealed variable efficiencies (fig. S10). AAV-Dmd CRISPR-injected muscles but none of the AAV-Ai9 CRISPR-injected muscles (Fig. 4D and fig. S10, F, H, and I). Quantification of exon 23 excision revealed variable efficiencies (fig. S10), which likely reflected targeting of only a subset of endogenous satellite cells that may be variably represented among the isolated and cultured cells. Finally, genomic PCR and amplicon sequencing confirmed targeted excision at the Dmd locus in satellite cell–derived myotubes (fig. S10K), and capillary immunostaining analysis revealed restored DYSTROPHIN expression (fig. S10L). As expected, injection of AAV-Dmd CRISPR did not induce tdtomato expression in satellite cells or myofibers of mdx;Ai9 mice (fig. S11).

In summary, this study provides proof-of-concept evidence supporting the efficacy of in vivo genome editing to correct disruptive mutations in DMD in a relevant dystrophic mouse model. We show that programmable CRISPR complexes can be delivered locally and systemically to terminally differentiated skeletal muscle fibers and cardiomyocytes, as well as muscle satellite cells, in neonatal and adult mice, where they mediate targeted gene modification, restore DYSTROPHIN expression, and partially recover functional deficiencies of dystrophic muscle. As prior studies in mice and humans indicate that DYSTROPHIN levels as low as 3 to 15% of wild type are sufficient to ameliorate pathologic symptoms in the heart and skeletal muscle (23–26) and that levels as low as 30% can suppress the dystrophic phenotype altogether (27), the restoration of DYSTROPHIN achieved here by one-time administration of AAV-Dmd CRISPR clearly encourages further evaluation and optimization of this system as a new candidate modality for the treatment of DMD (see supplementary text).
REFERENCES AND NOTES

16. Materials and methods are available as supplementary materials on Science Online.


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SUPPLEMENTARY MATERIALS

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Materials and Methods
Supplementary Text
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References (28–54)

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Fig. 4. Satellite cells in dystrophic muscles are transduced and targeted with systemically disseminated AAV-CRISPR.
(A) Experimental design. (B) Percentage of ZsGreen+ satellite cells expressing tdTomato after intraperitoneal injection of PAX7-ZsGreen(+):mdx;Ai9 mice. Individual data points overlaid with mean ± SD; vehicle (n = 3), AAV-Cre (n = 4), AAV-Ai9 CRISPR (n = 5). (C) Representative immunofluorescence of myotubes differentiated from FACS sorted satellite cells from mice injected intraperitoneally with vehicle, AAV-Cre, or AAV-Ai9 CRISPR. Green, myosin heavy chain (MHC); red, tdTomato; blue, DAPI (nuclei). Scale bar, 200 μm. (D) Exon 23-deleted Dmd mRNA in satellite cell–derived myotubes from mice previously injected intraperitoneally with AAV-Dmd CRISPR (right lanes), compared with control AAV-Ai9 CRISPR (left lanes).

AAV-Cre or AAV-CRISPR injection

Isolation of Pax7-Zsgreen+ cells from muscle

Analyzing myotubes by immunofluorescence and qPCR

Pax7-Zsgreen+/−:mdx;Ai9 mice

% tdTomato in satellite cells

Vehicle

Cre

SaCas9 + A19 gRNAs

MHC

tdTomato

Vehicle

Cre

SaCas9 + A19 gRNAs

MHC

tdTomato

D

AAV gRNAs

Dmd gRNAs

14 days

24 days

Expansion and myogenic differentiation of Pax7-Zsgreen+ cells

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Editing can help build stronger muscles
Much of the controversy surrounding the gene-editing technology called CRISPR/Cas9 centers on the ethics of germline editing of human embryos to correct disease-causing mutations. For certain disorders such as muscular dystrophy, it may be possible to achieve therapeutic benefit by editing the faulty gene in somatic cells. In proof-of-concept studies, Long et al., Nelson et al., and Tabebordbar et al. used adeno-associated virus-9 to deliver the CRISPR/Cas9 gene-editing system to young mice with a mutation in the gene coding for dystrophin, a muscle protein deficient in patients with Duchenne muscular dystrophy. Gene editing partially restored dystrophin protein expression in skeletal and cardiac muscle and improved skeletal muscle function.

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