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Spotlight

Exon Snipping in Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a life-limiting neuromuscular disorder caused by mutations in the *DMD* gene encoding dystrophin. We discuss very recent studies that used CRISPR/Cas9 technology to 'snip out' mutated exons in *DMD*, restoring the reading frame of the gene. We also present cautionary aspects of translating this exciting technology into clinical practice.

DMD is an X-linked disorder caused by mutations in the *DMD* gene and affecting approximately 1 in 3500 males. Different types of mutations ranging from deletions, duplications, point mutations, or other smaller gene rearrangements can generate an out-of-frame *DMD* transcript that leads to aberrant translation. Consequently, the protein dystrophin is absent. Dystrophin, a structural protein, provides a mechanical and signaling link between the actin cytoskeleton and the extracellular matrix in skeletal muscle [1]. The lack of dystrophin renders muscle cells susceptible to damage, leading to degeneration, loss of ambulation, respiratory weakness, and dilated cardiomyopathy in DMD patients.

By contrast, Becker muscular dystrophy (BMD) arises from deletions in which the *DMD* transcript is maintained in-frame, yielding a truncated, yet functional dystrophin that lacks the domains corresponding to exonic sequences affected by the mutation. Individuals affected with BMD show less severe clinical presentation than those with DMD, due to the ability of internally shortened dystrophin to partially fulfill its molecular function. Indeed, approximately 30% of dystrophin expression is deemed sufficient to prevent muscular dystrophy in humans [2].

The disparity in clinical severities between DMD and BMD has inspired the development of therapeutic strategies to modulate splicing patterns of the *DMD* gene, where antisense oligonucleotides (AOs) have been utilized to manipulate *DMD* pre-mRNA processing, and restore the *DMD* open reading frame to produce internally truncated dystrophin; a strategy known as 'exon skipping'. The AOs are complimentary to regions of the pre-mRNA, at, or in close proximity to the relevant *DMD* exon targeted for skipping. AO drugs targeting exon 51 have now been tested in advanced clinical trials [3,4] and are currently being considered for approval by regulatory agencies, such as the Food and Drug Administration.

An alternative approach to reframing the *DMD* gene has been offered by the discovery of clustered regularly-interspaced short palindromic repeats (CRISPR) technology, in which an endonuclease called Cas9 can cleave the genome in a precise manner when coupled with a strand of guide RNA (gRNA) [5]. The cleaved DNA will be rejoined via a non-homologous end joining (NHEJ) mechanism facilitated by the cells' own repair machinery or, alternatively, via homologous-directed repair (HDR), if a repair template is provided. While HDR is extremely inefficient in postmitotic cells such as skeletal muscle, NHEJ appears to be achieved in an efficient manner. Convincing evidence for the therapeutic potential of CRISPR/Cas9-mediated restoration of the *DMD* open reading frame, as we have dubbed 'exon snipping', has been recently reported by three independent groups who used dystrophin-deficient *mdx* mice (Figure 1) [6–8].

In the first study, Nelson *et al.* packaged *Staphylococcus aureus*-derived Cas9 in an adeno-associated vector serotype 8 (AAV8), as well as two gRNAs, targeted at introns 22 and 23 in another AAV8; they demonstrated that the intervening exon 23, which harbors a point mutation in dystrophin-deficient *mdx* mice, could be efficiently snipped out [6]. The resulting *DMD* transcript was in-frame, leading to restoration in the expression of dystrophin and components of the dystrophin glycoprotein complex. In addition, restoration of appropriate neuronal nitric oxide synthase (nNOS) localization and activity was achieved. Following systemic delivery, this approach was encouragingly efficient, rescuing dystrophin in abdominal, diaphragm, and cardiac muscles, yet not in distal limb muscles. Consequently, improved muscle motor function was not observed. As the authors stated in their conclusion, evaluation of various AAV capsids and tissue-specific promoters will be important for potential clinical translation of this approach.

This is partially addressed in the second study by Long *et al.*, who used an engineered AAV serotype 9-based vector containing *Streptococcus pyogenes*-derived Cas9 expressed under a minimal CMV promoter sequence, coupled to two gRNAs encapsulated in another AAV9 [7]. Following systemic administration, the reading frame and the expression of dystrophin in skeletal and cardiac muscles in *mdx* mice were successfully rescued. Improved grip strength activity was also observed. Importantly, dystrophin protein levels increased over time, which likely resulted from persistent expression of the CRISPR/Cas9

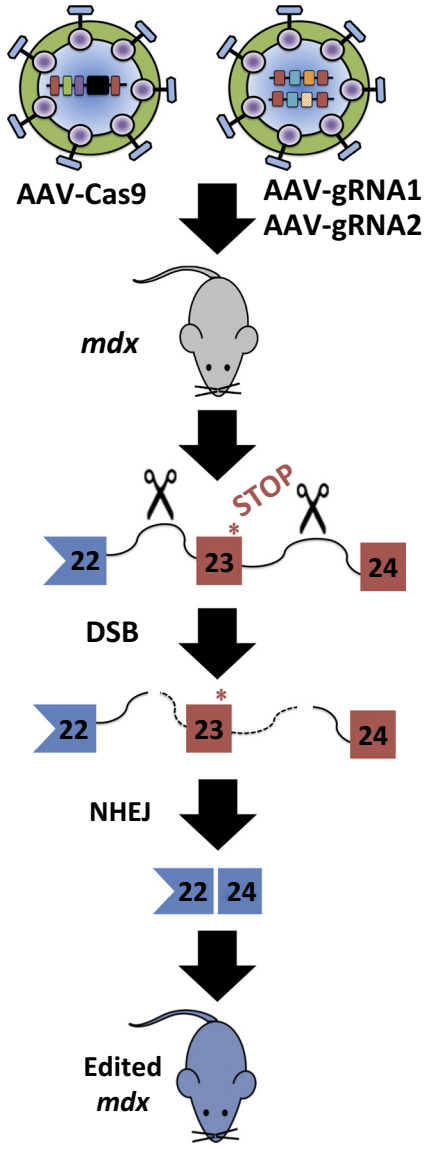

Figure 1. CRISPR/Cas9-Mediated Exon Snipping Strategy in *mdx* Mice. Adeno-associated vectors are used to deliver Cas9 in conjunction with guide RNAs (gRNAs) into dystrophin-deficient *mdx* mice, which harbor a point mutation (asterisk) in the *Dmd* exon 23, leading to a premature stop codon. Cas9 creates double-stranded breaks (DSBs) in introns 22 and 23 targeted by the gRNA1 and gRNA2, respectively, snipping out the flanking genomic sequence, including the mutated exon 23. The cleaved DNA is repaired via the mechanism of non-homologous end joining (NHEJ), and following pre-mRNA splicing, leads to an in-frame transcript comprising exons 22 and 24, restoring the dystrophin protein in the edited *mdx* mouse. Abbreviation: CRISPR, clustered regularly-interspaced short palindromic repeats.

components in postnatal skeletal muscles and/or a selective advantage of 'edited' muscle precursor cells.

Dystrophic muscles degenerate early and rapidly in response to the disease. Muscle resident stem cells, called satellite cells, are responsible for generating precursor cells that eventually arise to become mature muscle fibers and, therefore, are important targets for intervention. The third study by Tabebordbar *et al.* showed that in addition to mature muscle fibers, the CRISPR/Cas9-mediated dystrophin restoration could be efficiently achieved in satellite cells without hampering their regenerative capacity [8].

Collectively, these studies have effectively demonstrated that CRISPR/Cas9 technology can be delivered to terminally differentiate skeletal muscle fibers, cardiomyocytes, and muscle satellite cells in neonatal as well as adult mice. In addition, the system can be used to mediate targeted deletion of the mutation-containing exon, restore dystrophin expression, and recover functional deficiencies of dystrophic muscle, providing a critical proof-of-concept study to move this technology closer to the clinic. Moreover, all studies reported minimal off-target activity in the mouse genome.

Because of its sequence-specific nature, this strategy is promising. However, the absence of off-target activity in the mouse genome may not necessarily translate to patients with mutations in the dystrophin gene. Moving forward, the efficacy of 'exon snipping' needs to be explored in relevant patient cells, followed by a comprehensive, unbiased, genome-wide assessment of the off-target effects in humans. This type of assessment may provide relevant justification to further explore the therapeutic potential of this technology for DMD. Overall, it will be important to develop a framework that establishes guidelines and provides a basis for the development of clinically safe CRISPR/Cas9 treatments.

Nevertheless, the 'snipping' approach using two gRNAs has shown to be successful in deleting exons covering the mutation 'hotspot' region in the *DMD* gene, for example, exons 45–55, which would be applicable in treating 40–45% of DMD populations [9]. Furthermore, the technology can also benefit patients with duplications that comprise approximately 12–15% of the DMD mutation spectrum. Previous work from our laboratory has shown that duplication of exons 18–30 can be successfully removed in MyoD-converted

patient fibroblasts, leading to restoration of a full-length dystrophin protein [10]. Importantly, given the head-to-tail orientation of the duplicated region, one gRNA was sufficient to remove the mutation, which could prove to be beneficial, given the packaging limitation of an AAV vector.

The use of AAV vectors as a gene delivery method has tremendous potential in the gene therapy field. Several clinical trials pertaining to AAV9-mediated gene transfer in skeletal muscle for Pompe disease (NCT02240407) and spinal muscular atrophy (NCT02122952) are currently ongoing. In the three recent studies utilizing AAV vectors to deliver the CRISPR/Cas9 components discussed earlier, one-time injection led to dystrophin restoration up to 14 weeks. But it remains to be determined whether this therapy will need to be readministered after a certain period of time to maintain therapeutic levels of dystrophin. Administration of AAV-based gene therapies can be associated with a capsid-specific immune response, for example, production of neutralizing antibodies, which may nullify AAV present in the circulation and reduce the effectiveness of the vector, especially if readministration is required. Furthermore, Cas9 is a protein derived from a bacterial system, for which the extent of its immunogenicity in humans is still unknown. Preclinical studies addressing these potentially challenging immunological hurdles will be essential to propel these therapies into the clinic.

A positive aspect to consider is that the versatility of this technology allows editing to be implemented in virtually all DMD-causing mutations, providing tremendous potential for individualized treatment of DMD patients. One important question that requires thorough evaluation by regulatory agencies and industry is whether extensive preclinical assessment would be necessary for each gRNA designed. Creating animal models for each individual mutation to perform preclinical assessment is costly, time-consuming, and thus largely impractical. Patient-derived cells, by contrast, are easily obtained and may be sufficient to assess the efficacy of this method and its off-target activities. In addition, the development of constructs with tissue-specific promoters would be advantageous in reducing off-target effects, as well as minimizing or eliminating concerns of potentially targeting the germline/embryonic stage.

Taken together, the demonstration that CRISPR/Cas9-mediated genome editing can occur *in vivo* and lead to functional benefits in the dystrophin-negative *mdx* mouse provides a critical and exciting proof-of-concept that is necessary to take further steps into fine-tuning the development of this technology in adult somatic cells, and regard it as a potential promising treatment for DMD.

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