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REVIEW

Genome- and Cell-Based Strategies in Therapy of Muscular Dystrophies

Y. Bou Saada[1#], Carla Dib[1#], M. Lipinski[1], and Y. S. Vassetzky[1,2]*

[1]*UMR 8126, CNRS, Université Paris-Sud, Université Paris Saclay, Institut de Cancérologie Gustave-Roussy, F-94805 Villejuif, France; E-mail: vassetzky@igr.fr*
[2]*Lomonosov Moscow State University, 119991 Moscow, Russia*

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Abstract—Muscular dystrophies are a group of heterogeneous genetic disorders characterized by progressive loss of skeletal muscle mass. Depending on the muscular dystrophy, the muscle weakness varies in degree of severity. The majority of myopathies are due to genetic events leading to a loss of function of key genes involved in muscle function. Although there is until now no curative treatment to stop the progression of most myopathies, a significant number of experimental gene- and cell-based strategies and approaches have been and are being tested *in vitro* and in animal models, aiming to restore gene function. Genome editing using programmable endonucleases is a powerful tool for modifying target genome sequences and has been extensively used over the last decade to correct *in vitro* genetic defects of many single-gene diseases. By inducing double-strand breaks (DSBs), the engineered endonucleases specifically target chosen sequences. These DSBs are spontaneously repaired either by homologous recombination in the presence of a sequence template, or by nonhomologous-end joining error prone repair. In this review, we highlight recent developments and challenges for genome-editing based strategies that hold great promise for muscular dystrophies and regenerative medicine.

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Many genetic diseases still do not have curative therapies. Hence, genome editing as a novel strategy is a must to improve therapeutic implications. Genome editing utilizes engineered nucleases to discard, substitute, or incorporate DNA into a genome. Cells can be repaired *ex vivo* and then delivered into the body, or repaired *in vivo* where genes can be changed in cells still in the body. The repair is based on two steps: (i) introducing a double-strand break (DSB) in the desired location in the genome by nucleases; (ii) repairing the DSB making use of the cellular DNA repair machinery.

INTRODUCTION OF SITE-SPECIFIC DOUBLE-STRAND BREAKS

Engineered nucleases are now divided into four classes: meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas system (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated nucleases).

Meganucleases have been found in bacteria, eukaryotes, and Archaea [1]. These homing endonucleases (HEs) generate DNA DSBs at a determined locus using their sizeable cut domain (14-40 bp) [2]. Meganucleases are characterized by a strict DNA sequence identification, which makes them less toxic than other endonucleases like ZFNs. Despite the high number of discovered HEs, they

Abbreviations: Cas, CRISPR-associated nucleases; CMD, congenital muscular dystrophy; CRISPR, clustered regularly interspaced short palindromic repeats; crRNA, mature CRISPR RNA; DMD, Duchenne muscular dystrophy; DSB, double-strand break; FSHD, facioscapulohumeral muscular dystrophy; HDR, homology directed repair; INDEL, insertion or deletion; iPSC, induced pluripotent stem cells; NHEJ, nonhomologous end joining; ODN, oligodeoxynucleotide; PAM, protospacer adjacent motif; PNA, peptide nucleic acids; rAAV, recombinant adeno-associated virus; RVD, repeat-variable diresidues; TALEN, transcription activator-like effector nuclease; tracrRNA, transactivating crRNA; ZFN, zinc finger nuclease; ZFP, zinc-finger protein.

# These authors contributed equally to this work.
* To whom correspondence should be addressed.

are sequence specific-endonucleases and unable to complement all the variety of genome sequences, which decreases the chance of finding an analogous locus in a target gene. This led many researchers to engineer specific endonucleases using mutagenesis and high-throughput screening methods [3, 4]; however, creating HEs for all genome sequences is costly and time consuming. Moreover, chromatin structure strongly influences access to its recognition sites [5]. Indeed, the efficacy of 42 newly generated nucleases at targeted mutagenesis was not correlated with their activity in transiently transfected cells, suggesting that epigenetic mechanisms control the efficacy of genome editing [3].

Zinc finger nucleases result from the fusion of zinc-finger proteins (ZFPs) to the FokI nuclease cleavage domain [6]. FokI is a bacterial enzyme that contains two domains: the first ensures the binding to DNA, and the second catalyzes cleavage. The FokI cleavage site requires dimerization [7, 8] and the binding of the endonuclease to the DNA to cut it upstream from the recognition site, without additional sequence particularity [9]. Since FokI is a nonspecific nuclease, engineering of ZFNs that are able to target specific sites in the genome is totally determined by the efficacy of the created ZFPs.

The ZF (zinc finger) motif contains ~30 a.a. that form a $\beta\beta\alpha$ structure; the α-helix insures the DNA binding of ZF motifs via its insertion to the major DNA groove [10]. Theoretically, each "finger" binds to three nucleotides. Six amino acids of each ZF motif play a main role in the specific recognition of the DNA target sequence [10-12]. Therefore, to create ZF motifs, these amino acid residues can be changed, while the rest can be kept as a backbone [13, 14]. The binding of two ZFN "arms" to the analogous DNA sequence permits FokI activation by dimerization. Studies have demonstrated a lack of specificity of ZFNs. In fact, engineered ZFNs were able to cut into a trinucleotide repeat region by a simple homodimerization using just one ZFN "arm" [15-17]. To avoid the possibility of undesirable homodimer activity and to reduce occurrences of genome-wide off-target cleavage, new ZFs with four fingers instead of three and a new FokI architecture, which only functions as an obligate heterodimer, have been engineered [18].

Similarly to ZFNs, TALENs are based on a nonspecific DNA cutting enzyme, the FokI nuclease domain, linked to transcription activator-like effectors (TALEs), a precise DNA sequence identifying amino acids [19]. TALEs have been found in the plant pathogen *Xanthomonas* [20]. Their DNA-binding domain is constituted of several repeats of ~34 a.a.; these repeats are identical for all the residues except the 12th and 13th ones, which are variable in each repeat. Studies have decoded a correlation between the repeat-variable diresidues (RVD) sequence and target DNA bases. In fact, each RVD recognizes more or less specifically the four DNA bases [21, 22]. The TALEs repeated models constitute a benefit to these nucleases, they permit the designing of a large number of specific proteins by simply changing the repeats combinations.

In TALEN also, two arms should bind to the DNA target sequence to enable FokI catalyzing domain dimerization, and thus its activation. Although ZFNs and TALENs are comparable on the nuclease level, they have distinct peptide sequences and consequently different DNA recognition interactions. Each TALE DNA-binding domain recognizes one nucleotide, whereas ZFNs interact with trinucleotides, which makes it a more complex recognition. These two technologies can be used in a variety of organisms: ZFN and TALEN modifications have been used in yeast [23, 24], zebrafish [25, 26], fruit flies [27, 28], plants [29, 30], rats [31, 32], and in human cells [33-36].

The latest developed genome editing technology is the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated nuclease (Cas) system. CRISPR/Cas, discovered in bacteria and Archaea [37], is a form of acquired immunity that grants to prokaryotes the ability for defense against any foreign DNA [38-40]. The three CRISPR systems discovered to date are designated as type I, II, and III [41].

Type II, referred to as CRISPR, is the fundamental system for current genome-editing technology. It consists of a nuclease (Cas9) and two RNA molecules: a mature CRISPR RNA (crRNA) and a transactivating crRNA (tracrRNA) that couples with crRNA [42]. The crRNA targeting sequences are transcribed from DNA sequences known as protospacers [43]. Protospacers are short sequences (~20 bp) of previously encountered foreign DNA, separated by a short palindromic repeat and kept to confer protection against other possible invaders [44, 45]. These protospacers are clustered in the bacterial genome in a group called a CRISPR array. This array is transcribed into a long RNA precursor that is processed by the bacterial ribonuclease RNase III [46]. This produces short mature crRNA, each analogous to a specific foreign sequence [47, 48]. In fact, it is the tracrRNA hybridization to the short palindromic repeat that activates RNase III [46].

Later on, the CRSIPR system was modified by fusing both RNAs (crRNA and tracrRNA) into a single chimeric guide RNA (sgRNA or gRNA) executing both functions [49]. However, this genome engineering system has a critical limitation in application. Every potential genomic target sequence must have a short nucleotide sequence, termed a protospacer adjacent motif (PAM), on its 3′-end [49-52]. This condition makes the type II CRISPR system restricted to target sequences followed by NGG, the PAM sequence.

One of the most important advantages of CRISPR/Cas9 over the nuclease families mentioned above is the extreme simplicity of designing it, as only 20 nucleotides recognizing the targeted region need to be synthesized. Veres et al. showed that Cas9 has much high-

er efficacy than TALEN in a study where the *SORT1* human gene was targeted by both genome editing tools in human stem cells. Still, a very low number of unwanted mutations have been shown for both of the engineered nucleases [53].

DOUBLE-STRAND BREAKS AND THEIR REPAIR

Once the nuclease has created a DSB in the genome, cells employ two main mechanisms to repair it: nonhomologous end joining (NHEJ) and homology directed repair (HDR). NHEJ by directly joining the DNA ends in a DSB causes deletions and/or insertions at the target site. It was shown responsible for mutations in 50% of DSB at the repair site in mycobacteria [54]. NHEJ may also generate dangerous chromosomal translocations (reviewed in [55]). However, HDR can be exploited to limit sequence changes by introducing an exogenous template that includes the desired sequence change into the same cell along with the nuclease, and this DNA template will trigger HDR-specific and precise modifications at the targeted locus.

MUSCULAR DYSTROPHIES

Myopathies can be either genetically inherited or occur because of endocrine defects, inflammation, or other abnormalities. Some are classified as single gene disorders, and they are the most amenable to gene therapy among other genetic disease categories.

Muscular dystrophies (MDs) constitute a class of myopathies that occur by mutations affecting genes having a critical role in skeletal muscle. These gene alterations are often the origin of skeletal muscle loss and weakness.

Duchenne muscular dystrophy (DMD) is a severe inherited disorder triggered by mutations of a gene that codes for dystrophin [56]. These mutations can also occur in a sporadic way, constituting a third of cases [57, 58]. Dystrophin has an important role as it links the muscle cell cytoskeleton to the adjacent extracellular matrix [59]. Men are predominantly affected in DMD because it is an X-linked recessive disease.

The dystrophin gene is also affected in Becker muscular dystrophy (BMD), but in this disorder the mutated gene generates a shortened protein that is still partially functional, which makes BMD a less grave disorder than DMD [60]. Dystrophin is a component of a protein complex. Alterations affecting genes encoding components of this dystrophin–glycoprotein complex generate congenital muscular dystrophy (CMD) and limb-girdle muscular dystrophies.

Many types of congenital muscular dystrophies (CMD) probably arise due to alterations in α-dystroglycan glycosylation, which is a component of the dystrophin complex. This complex has a crucial role in transmembrane linkage between muscle cell cytoskeleton and the basal lamina [61]. Different types of CMDs are classified based on the defects occurring in these disorders. They could be alterations of glycosylation, mutations of genes encoding for structural muscular proteins, or the endoplasmic reticulum proteins and mitochondrial membrane proteins [62, 63].

Myotonic dystrophy (DM) is an autosomal-dominant disorder characterized by multisystemic clinical features. Two types of this myopathy, DM1, termed Steinert's disease, and DM2 result from expansion of trinucleotide repeats. In the first type, the alteration occurs in the *DMPK* gene [64] that maps to the long arm of chromosome 19 [65]. The *DMPK* gene encodes a protein expressed mainly in skeletal muscles [66]. Whereas in proximal myotonic myopathy (PROMM), the DM2 type, it is the *ZNF9* (zinc finger protein 9) gene on chromosome 3 that is affected [67]. Both DM types have similarities in clinical symptoms such as diabetes, muscular weakness, and cardiac failure [68, 69].

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant neuromuscular disease with prevalence that can reach 1 in 8000 [70]. Clinically, FSHD is characterized by a progressive weakness and atrophy of facial muscles and the shoulder girdle. The major genetic form of FSHD has been mapped to the subtelomeric region of the long arm of chromosome 4 [71]. In this region, three abnormalities have been specifically associated with FSHD: a partial deletion within D4Z4, a polymorphic macrosatellite repeat array; the presence of SSLP-161, a specific simple sequence length polymorphism; and that of the 4qA allele [72]. This three-feature combination causes chromatin organization modification in the 4q35 chromosomal region in FSHD patients [73-76], which releases the inhibition otherwise imposed on the expression of *DUX4*, a gene contained in the D4Z4 repeat. This change in chromatin organization possibly also leads to the overexpression of *ANT1*, *FRG1*, *DUX4c*, and *FRG2*, four genes positioned centromerically to D4Z4, and each has been implicated in FSHD [77-80].

Other disorders classified as muscular dystrophies subgroups like distal muscular dystrophy (DMD) [81], Emery–Dreifuss muscular dystrophy (EDMD) [82], limb-girdle muscular dystrophy (LGMD) [83], and oculopharyngeal muscular dystrophy (OMD) [84] also arise due to gene defects.

DEFINITIONS OF GENE AND CELLULAR THERAPIES

Many strategies of gene therapy have been tested trying to modify expression of genes of interest to trigger

alterations of certain biological functions. Gene therapy, initially devised by Friedmann and Roblin in 1972 [85], is defined as the delivery of nucleic acids, including DNA, RNA, and their analogs to cells of a living organism to treat diseases via the use of viral, nonviral, or cell-based vectors.

Cell therapy is a form of gene therapy itself, if a functional gene of natural or exogenous origins is delivered using cells as vectors. In fact, cell therapy is based on the delivery of precursor cells that are supposed to regenerate muscle fibers in myopathies.

GENE AND CELLULAR THERAPY IN MUSCULAR DYSTROPHIES

So far, there is no cure for most muscular dystrophies, but some gene and cellular therapy methods have been explored to treat different types of MDs to express a defective gene or inhibit the expression of a faulty gene.

In Duchenne muscular dystrophy (DMD), the patients lack dystrophin, so the main goal of its therapy is to express this crucial muscle protein. One approach that has been exploited by researchers consists of using molecules that prompt exon skipping. Patients with DMD caused by RNA reading frame mutations have shown clinical improvement using these antisense oligonucleotides. With this approach, a shortened but more functional dystrophin protein is produced [86, 87]. Other antisense oligonucleotides have been evaluated and showed some dystrophin expression repair in patients' muscles (NCT01803412, NCT02255552).

Recombinant adeno-associated viruses (rAAVs) have also been investigated for gene addition in DMD animal models. rAAVs have the promoter/transgene of interest bordered by the viral inverted terminal repeats (ITRs) and all viral genes discarded. Even though rAAV vectors have demonstrated limited immunogenicity in small animal studies, it has been shown that they can elicit variable immunological outcomes in large animal models [88, 89]. However, transient immunosuppression effect has been evaluated in canine models and demonstrated micro-dystrophin expression for several months [90, 91]. rAAV8 vector systemic and intramuscular delivery allowed robust dystrophin expression in muscles of macaque models, although the outcome efficiency was affected by preexisting AAV8 antibodies [92].

In a rAAV trial for DMD, patients received AAV2.5 CMV microdystrophin to avoid preexisting antibodies. AAV2.5 is a variant of AAV2 that retains the properties of easy purification from AAV2 and improvement in transduction efficiency in muscle from AAV1. Nevertheless, patients who had preexisting immunity to AAV2 and were able to neutralize rAAV2.5 showed fewer detected vector genomes than other patients did. Moreover, dystrophin-specific T-cells were detected in four patients; this may provide an explanation for the low detected levels of dystrophin expression [93].

In *mdx* mice (a mouse model of DMD), promising work has been done: intramuscular delivery of normal mouse donor myoblasts converted dystrophic *mdx* myofibers from dystrophin-negative to dystrophin-positive [94], but clinical trials of transplantation of myoblasts in DMD patients have shown discouraging results [95-97]. They failed to provide the patients any sustained clinical benefit for several reasons, including low survival and migration from the injection site of grafted myoblasts and immunological rejection by the host [98]. The objective was then to find a cell that harbors myogenic potential, can be systemically delivered, and functionally reconstitute the muscle stem cell niche to provide long-term production of dystrophin-positive muscle fibers. Pericytes seem to be the most promising cell type for DMD cell therapy. Delvalle et al. showed that human muscle-derived pericytes promote significant muscle regeneration in immunodeficient, dystrophin-deficient mice (SCID mdx) [99].

A gene therapy trial for limb-girdle muscular dystrophy type 2D (LGMD2D) was carried out in the US by Professor Jerry Mendell. In this trial, an adeno-associated viral vectors (AAV1) virus containing a functional α-sarcoglycan gene, which is affected in this myopathy, was injected into a muscle in the patients' feet. The injected muscles generated the alpha-sarcoglycan protein in many muscle fibers [100] without evidence of cytotoxicity. These findings are very promising, but systemic delivery of the healthy gene copy is required for improvement in symptoms to be seen in patients. Liu et al. carried out a preclinical trial in LGMD1A mice targeting mutant myotilin (MYOT), the principal cause of LGMD type 1A [101]. Therapeutic microRNAs containing AAV6 that were used reduced significantly the level of altered myotilin mRNA and the respective generated protein [102].

Based on the consensus model of facioscapulohumeral dystrophy pathophysiology, most of the proposed therapeutic approaches aim to repress the *DUX4* gene by targeting DUX4 mRNA, DUX4 protein, or DUX4-induced processes implicated in FSHD. Wallace et al. adopted a gene silencing approach utilizing RNA molecules that inhibited *DUX4*. This led to correction of DUX4-associated-myopathy in mouse muscle [103]. Even in mice over-expressing FSHD region gene 1 (FRG1), the RNA interference approach demonstrated amelioration in myopathic phenotypes [104, 105].

GENOME EDITING IN MYOPATHIES

Duchenne muscular dystrophy. DMD is the most common myopathy and one of the most recessive disorders in the human population, with incidence of 1 : 5000

[106]. DMD is a recessive X-linked disorder with symptoms appearing in boys at the age of approximately four. It is characterized by progressive muscle wasting and weakness, most DMD patients becoming wheelchair-dependent and ventilation-assisted. Due to its aggressiveness, DMD is lethal, and death occurs between the second and fourth decade of life. The dystrophin (*DMD*) gene was mapped to the short arm of the X-chromosome (RefSeq NG_012232.1); the locus covering approximately 1% of the X-chromosome is large and is thus subject to deletion mutations predominantly (65%) or duplications (11%) and to a lesser extent for mutations affecting the coding sequence and splice sites. The resulting out-of-frame mutations or nonsense mutations found in DMD patients result in prematurely truncated and nonfunctional dystrophin protein [107].

Efforts have been made to develop animal models carrying the genetic mutation associated with DMD and recapitulating the disease pathophysiology; more than 60 dystrophin-deficient animal models have been developed including rodent models [108-112], canine models [113, 114], a porcine model [115], and non-mammalian models such as *dmDys Drosophila* [116], zebrafish [117], and *Caenorhabditis elegans* [118].

Several therapeutic approaches have been extensively investigated, from cellular and stem cell therapies to pharmacological strategies and gene therapy. Currently, stem cell therapy studies are challenging and are being optimized to ameliorate the stem cell isolation, culture, and delivery on one hand, and to improve muscle quality and environment on the other hand. Dystrophin-based gene therapies consist of the addition of the dystrophin gene. Due to difficulties regarding the delivery of the large-sized dystrophin gene into myogenic cells and with the discovery and development of genome editing tools, a new era of gene therapy for DMD was opened, and the possibility of permanently correcting the *DMD* gene became viable.

Genome editing for DMD using single-stranded oligonucleotides. A number of strategies have focused on editing the dystrophin gene. Notably, Bertoni et al. employed an oligonucleotide-mediated genome repair strategy where an oligonucleotide vector containing single base-pair alterations specifically targeting the genome sequence of interest, creating a mismatch that is then repaired by the endogenous DNA repair mechanisms with the probability of single-base changes in the dystrophin gene sequence [119]. By using chimeric RNA/DNA oligonucleotides (RDOs) and oligodeoxynucleotides (ODNs), point mutations were successfully corrected in *in vitro* and *in vivo* studies. Later Maguire et al. repeated the correction of the dystrophin gene by means of modified single-stranded oligonucleotides coupled with an RNAi against an inhibitor of gene repair, Msh2, which increased the level of gene repair in myoblasts [120]. In another approach, Kayali et al. designed single-stranded ODNs made of peptide nucleic acids (PNAs) bases that are DNA mimics having a stronger binding affinity to DNA than that of DNA–DNA interaction. These PNA-ssODNs harboring single mismatches were able to induce stable alterations in the dystrophin gene and to subsequently restore dystrophin protein expression *in vitro* and *in vivo* [121]. All these studies focused on correcting muscle cells *in vitro* and correction of mature myofibers in treated mice. These approaches were not able to provide long-term dystrophin expression; it was not sufficient to protect muscles from degeneration. To overcome this limitation, PNA-ssODNs targeting the dystrophin gene were later successfully tested on satellite cells isolated from a DMD mouse, and when transplanted into skeletal muscle of dystrophin-deficient mice, the corrected-satellite cells had long-term ability to restore dystrophin expression in myofibers [122].

Genome editing for DMD: from meganucleases to CRISPR-Cas9 technologies. Many scientists have focused particularly on DMD as a model for myopathies associated with single-gene mutations and loss of function. By inducing site-specific double-strand breaks (DSBs), endonucleases increase the rate of homologous recombination (HR) up to 10,000-fold *in vitro* [123, 124]; thus, several gene correction modes can be considered: gene correction by insertion of the correct sequence (template), induction of exon skipping by introducing mutations at splice sites, and removal of exons [125, 126].

Rousseau et al. tested the relevance of using meganucleases and zinc-finger nucleases for targeting and introducing INDELs (insertions or deletions) in different introns and in exon 50 of the dystrophin gene [127]. The mutations frequency was 10- and 300-fold more abundant in 293T cells transfected with dystrophin-targeting meganucleases and ZFNs, respectively, as compared to mock-transfected cells. They were also tested on human primary myoblasts, and ZFNs induced 30 times more INDELs than mock-transfected myoblasts. Interestingly, the rate of INDELs was increased when ZFN-transfected primary myoblasts were induced to differentiate into myotubes, which triggers the expression of the dystrophin gene. In this study, Rousseau et al. were able, by means of meganucleases and ZFNs, to induce INDELs by triggering NHEJ-based DSB repair, with some of these INDELs indirectly changing the reading frame of the dystrophin gene. Although the efficiency was low, these promising results created solid ground for further utilization of genome editing tools for optimizations and experiments.

In 2013 Ousterout et al. investigated the use of TALENs to restore the reading frame of the dystrophin gene by introducing INDELs into exon 51 and inducing frameshifting by TALEN-mediated NHEJ in human immortalized DMD cells [128]. They successfully isolated clonal populations of myoblasts harboring corrected dystrophin gene and producing, once differentiated, the dystrophin protein. Interestingly, the introduction of

TALEN in DMD patient-derived primary dermal fibroblasts carrying exon 46-50 deletion efficiently led to gene modification and frameshifting, and when these corrected dermal fibroblasts were induced to transdifferentiate into the myogenic lineage, the expression of dystrophin was rescued, similarly to corrected human immortalized DMD myoblasts. This approach could be interesting for cell-based therapies since MyoD-transduced dermal fibroblasts are investigated for *ex vivo* gene therapy of other myopathies [129].

Later, a study from Dickson's team proved for the first time the feasibility of meganuclease-dependent knock-in for correcting the dystrophin gene in *in vitro*-cultured human DMD myoblasts [130]. This study focused on targeting intron 44 of the dystrophin gene in human DMD myoblasts carrying exon 45-52 deletion (del45-52 DMD cells), since 25% of the mutations causing DMD arise from this intron. Specific I-CreI-meganuclease and repair template with long arms of homology were designed and cloned into lentiviral vectors. The meganucleases successfully induced a DSB at the specific target site on intron 44 in del45-52 DMD myoblasts, and when the cells were co-transduced with the lentiviral vector containing the repair matrix, they successfully produced a corrected dystrophin transcript harboring exon 45-52 cDNA. This successful knock-in approach based on specific homologous recombination proved the feasibility of this genome editing strategy applied on *in vitro* primary DMD myoblasts, the dystrophin gene being permanently corrected, but the production of dystrophin protein remained to be verified.

Another genome editing strategy was then described by Ousterout et al. [126] that is aimed at permanently skipping exon 51 and restoring the reading frame of dystrophin gene, similar to the oligonucleotide-mediated exon skipping approach, which concerns around 13% of all DMD deletions. In this study, a pair of ZFNs was designed to target two sites flanking the exon 51 splice acceptor sequence, thus to permanently delete exon 51 from the mRNA transcript in DMD myoblasts. Instead of introducing random INDELs leading to unpredictable changes in the final protein, this strategy has the advantage of introducing specific and nonrandom INDELs by the means of two ZFNs, resulting in the loss of exon 51 and precise and reproducible frameshifts. Thus, these nonrandom events result in a predictable function of derived-dystrophin transcript. Indeed, transplantation of these genetically corrected DMD myoblasts into immunodeficient mice led to dystrophin expression at the sarcolemma membrane of muscle cells.

With the validation of TALENs and CRISPR-Cas9 effectiveness for gene correction in human induced pluripotent stem cells (iPSCs), Li et al. conducted a study for targeting and correcting dystrophin gene mutation in DMD in iPSCs [125]. The advantage for working on iPSCs is their unlimited self-renewal capacity and their ability to differentiate into myoblasts, which offer a good model for eventual autologous transplantation. They authors generated iPSCs derived from a DMD patient lacking exon 44 and applied three different approaches for restoring the dystrophin protein using their own designed TALENs and CRISPR-Cas9: exon 45 skipping, frameshifting by small INDELs, and exon 44 knock-in. All three approaches worked successfully on patient-derived iPSCs with both TALENs and CRISPR-Cas9, with no severe off-target mutagenesis. By differentiating corrected iPSC clones into skeletal muscle cells, the dystrophin protein expression was restored, with restoration of the full amino acid sequence of dystrophin only in knocked-in clones.

With the emergence and expansion of CRISPR-Cas9 in the field of genome editing, Ousterout et al. took advantage of the multiplex gene editing capability and facility of this system to target multiple genes simultaneously [131, 132]. They successfully targeted exons 45-55 of the dystrophin gene and induced frameshifts and large deletions covering common mutational hotspots found in more than 60% of all DMD patients [133]. First, they used a multiplex CRISPR-Cas9 system to delete exon 51 in DMD myoblasts by introducing Cas9 along with two sgRNAs flanking exon 51; this genomic deletion restored dystrophin expression at the RNA and protein levels in differentiated corrected-myoblasts. In a second approach, Ousterout et al. focused on creating a multiplexing CRISPR-Cas9 system in which exons 45-55, a region containing more than 60% of DMD patients' mutations, are entirely deleted. Interestingly, this multiplexing system led to a large genomic deletion of 336 kb, excluding exons 45-55 region, and it was accompanied by restored dystrophin mRNA and protein expression. Although a major concern of this approach is the increased probability of chromosomal rearrangements due to the multiple induced DSBs, this single genome editing strategy offers a solid ground for gene therapy applicable for hereditary diseases with single- or multiple-mutations.

The first use of the CRISPR-Cas9 editing system in the germline of *mdx* mice was recently described [134]. To rescue respiratory, cardiac, and skeletal muscles and to encounter challenges regarding *in vivo* delivery systems, Long et al. demonstrated the feasibility of genetic repair in the germline of dystrophin-deficient mice by co-injecting Cas9, sgRNA, and a repair-template into mouse zygotes, hoping to correct dystrophin mutation in all cells of a *mdx* mouse harboring a nonsense mutation in exon 23. This strategy resulted in a wide range of genetically mosaic mice containing 2 to 100% correction of the dystrophin gene, since genome editing can occur in a subset of embryonic stem cells. Interestingly, only 17% of gene correction is sufficient to obtain a level of dystrophin expression in myofibers similar to that of wild-type mice, and to prevent muscular dystrophy.

More recently, three research teams simultaneously tested the CRISPR-Cas9 genome editing system on a

postnatal mouse model of DMD; the dystrophin reading frame was rescued, and dystrophin protein expression was restored in cardiac and skeletal muscle after the delivery of AAV9 carrying gene editing components into postnatal *mdx* mice. Dystrophin expression increased with time and was accompanied with enhanced skeletal muscle function [135-137].

These studies demonstrated the large and variable options scientists have when manipulating genome editing tools. Endonucleases can be designed to specifically target every mutation causing DMD, thus giving hope to correct all forms of DMD. The majority of studies focused on proving the feasibility of genome editing primarily for treating DMD. Genome editing tools are driving innovative applications from basic biology to medicine, and advances in correcting genetic defects of other dystrophies have been made.

Genome editing in other dystrophies. *Spinal muscular atrophy* (SMA) is an autosomal recessive genetic disease originating from alteration of the survival motor neuron 1 (*SMN1*) gene, resulting in progressive loss of motor neurons and progressive muscle wasting characteristic of SMA patients. To recover motor neuron loss, an ssODN targeting the *SMN2* gene, a gene paralog to *SMN1*, was able to redirect its splicing. Thus, the resulting protein is similar to SMN1 and is able to partially rescue motor neuron loss and to ameliorate defects in neuromuscular function in a SMA mouse model [138].

Facioscapulohumeral dystrophy. In vertebrates, the muscle differentiation process is highly regulated and is activated when muscle tissue damage occurs. This requires proliferation and fusion of muscle satellite cells and implies the coordination and regulation of several pathways by MyoD, Myf5, and specific miRNAs expression [139].

Myoblasts from FSHD patients exhibit defects in their morphological differentiation. Unlike normal myoblasts that fuse and form branched myotubes with aligned nuclei, FSHD myotubes are either atrophic, or disorganized with a random distribution of nuclei [140]. A recent study from our laboratory successfully tested the feasibility of cell therapy in FSHD by correcting these defects at the morphological and functional levels by adding normal myoblasts in the FSHD cell culture in different proportions, and inducing myogenic differentiation *in vitro*. The number of hybrid myotubes with normal phenotype was directly linked to the amount of normal myoblasts initially present in the myoblast mix. The presence of >50% of normal myoblasts led to hybrid myotubes mostly with normal phenotype. We also observed the normalization of the transcription profile of the hybrid myotubes [141].

A recent work reported the use of the nuclease-deficient CRISPR-dCas9 system to target and manipulate gene expression in *in vitro* cultured myocytes derived from FSHD patients. The catalytically inactive dCas9 does not generate double-strand breaks, but it can be fused to transcriptional regulators that can be therefore brought to the target locus. Targeting transcriptional effectors fused to CRISPR-dCas9 to the DUX4 promoter resulted in enhanced chromatin repression of the FSHD pathogenic locus and a decrease in DUX4 expression [142].

GENOME EDITING IN MYOPATHIES: LIMITATIONS AND CHALLENGES

Although proof-of-concepts and encouraging results have been obtained with genome editing tools studied on *in vitro* systems and animal models, several limitations still have to be overcome before genome editing can be applied in the context of clinical applications. Whether correcting genetic mutations *ex vivo* or specific cell type *in situ* remains the major question. The most challenging hurdles that need to be addressed are the limited level of gene editing frequencies, the risk of off-target mutations, the use of viral vectors to carry nucleases, the choice of an appropriate cell type in case of cell therapy, and the mode of delivery into the organism.

PERSPECTIVES

To date, the most promising and safest strategy is to correct the patient's muscle stem cells *ex vivo*. Although optimizing culture techniques to maintain their regenerative potential, characterization of their stem-cell properties and regulations, and additional parameters are prerequisite for using muscle stem cells for clinical applications, several discoveries and trials during the past decade have achieved great advances in the field of genome editing strategies in muscle stem cells.

Gene-editing technologies hold great promise for improving human health as they can be used to investigate new gene functions and regulations and they are capable of correcting or introducing point mutations and regulating transcription and epigenetics. They have also accelerated and expanded researchers' ability to generate genetic models. They can be used to directly treat deleterious genetic diseases in somatic cells. However, establishing appropriate delivery, specificity, and repair strategies are prerequisites for clinical use. Recently, a Chinese research team used CRISPR-Cas9 technology on human embryos and obtained mosaic embryos harboring a large number of off-targets that can be potentially harmful if the embryos were viable [143]. Social and ethical aspects for editing human embryos should be carefully taken into consideration.

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