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GENE AND CELL-MEDIATED THERAPIES FOR MUSCULAR DYSTROPHY

Patryk Konieczny, PhD^{1,2,*}, Kristy Swiderski, PhD^{1,3,*}, and Jeffrey S. Chamberlain, PhD¹

¹Department of Neurology, The University of Washington School of Medicine, Seattle, WA, USA, 98105

Abstract

Duchenne muscular dystrophy (DMD) is a devastating muscle disorder that affects 1 in 3500 boys. Despite years of research and considerable progress in understanding the molecular mechanism of the disease and advancement of therapeutic approaches, there is no cure for DMD. The current treatment options are limited to physiotherapy and corticosteroids, and although they provide a substantial improvement in affected children, they only slow the course of the disorder. On a more optimistic note, the most recent approaches either significantly alleviate or eliminate muscular dystrophy in murine and canine models of DMD and importantly, many of them are being tested in early phase human clinical trials. This review summarizes advancements that have been made in viral and non-viral gene therapy as well as stem cell therapy for DMD with a focus on the replacement and repair of the affected dystrophin gene.

Keywords

Duchenne muscular dystrophy; gene therapy; cell therapy; dystrophin; stem cells

Genetic diseases affect many individuals worldwide in all stages of life. These diseases can be classed as 1 of 4 categories: single gene disorders, multifactorial disorders, chromosomal disorders, and mitochondrial disorders. Of these, the single gene disorders are most amenable to gene therapy. Gene therapy describes the process of gene replacement or gene repair and can be performed either *in vivo*, where the gene is delivered to cells within the body, or *ex vivo*, where stem cells are genetically corrected and then delivered into the body. The latter may also be referred to as cell-mediated therapy. The earliest clinical gene therapy trials targeted cells of the hematopoietic system,^{1,2} but the focus has now broadened to a wide range of single gene disorders. The muscular dystrophies are among the most common single gene disorders afflicting the population. There are many different types of muscular dystrophy, which are caused by mutations in various genes that play functionally important roles in muscle. Duchenne muscular dystrophy (DMD), the most common form, afflicts 1 in 3500 newborn males. This review will discuss gene and cell-mediated therapies aimed at

Correspondence to: J.S. Chamberlain; jsc5@uw.edu.

²Current address: Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland

³Current address: Department of Physiology, The University of Melbourne, Victoria, Australia 3010

*The first two authors contributed equally to this work

treating DMD; however, most therapies described here may also be applicable to treatment of the other muscular dystrophies.

DUCHENNE MUSCULAR DYSTROPHY

DMD is a fatal, X-linked, recessively inherited disorder characterized by progressive muscle wasting that arises from mutations in the dystrophin (*dmd*) gene. The *dmd* gene encodes a 427 kDa protein termed dystrophin that links the actin cytoskeleton to the extracellular matrix in muscle fibers by forming interactions with subsarcolemmal actin and a multimeric protein complex termed the dystrophin-glycoprotein complex (DGC; Fig. 1).³⁻⁵ Absence of the dystrophin protein, such as occurs in DMD, weakens the link between the sarcolemma and the actin cytoskeleton, resulting in membrane instability and muscle cell death.

Skeletal muscles of DMD patients and of the murine (*mdx*) and canine (*cxmd*) models of DMD lack dystrophin, causing them to be more susceptible to eccentric contraction-induced injury.⁶ In response to such injury, damaged muscle fibers undergo repeated cycles of segmental necrosis and regeneration, during which satellite cells, the primary muscle stem cell, become activated to regenerate muscle fibers (Fig. 2).^{7,8} However, the regenerative process is largely inefficient and results in fibrotic changes and fatty deposition in skeletal muscle. The most severely affected skeletal muscle is the diaphragm, where muscle wasting contributes to respiratory failure in older patients and mice.⁹⁻¹¹ In conjunction with skeletal muscle pathology, DMD also affects cardiac muscle, and to a lesser extent, smooth muscle. Studies have revealed that more than 90% of DMD patients develop cardiomyopathy,¹²⁻¹⁵ and studies in the γ -sarcoglycan-deficient mouse (a mouse model of a related limb girdle muscular dystrophy) have revealed that skeletal muscle-specific re-expression of γ -sarcoglycan, where the cardiac muscle remains γ -sarcoglycan-deficient, results in declining function of the heart.¹⁶ This is true also for DMD,¹⁷ which highlights the importance for gene or cell-mediated therapies that target not only skeletal muscle but also the heart. It remains unclear how important dystrophin expression in smooth muscle cells is for patient quality of life.

GENE THERAPY FOR DMD

The muscular dystrophies, including DMD, are attractive candidates for gene therapy, as all types arise from single-gene mutations. When the dystrophin gene was first identified in 1987^{18,19} it was hoped that gene therapy for muscular dystrophy would follow soon after.²⁰ However, while many advances have occurred, the development of an effective gene therapy for DMD still faces significant challenges.²¹ These include determining the optimal mode of gene delivery, addressing the advantages and disadvantages of gene replacement versus gene repair, and overcoming the immune challenges presented not only by each technique, but also by the reintroduction of a gene that may be recognized as foreign by the immune system of DMD patients.

Gene therapy for DMD requires the delivery of a new dystrophin gene to all muscles of the body, which make up greater than 40% of the body mass, including the diaphragm and the heart. Investigations have revealed that many symptoms of the disease, such as high creatine kinase levels, fiber degeneration, and inability to generate force are prevented in *mdx* mice

that express as little as 20% of the wild-type dystrophin levels, although the level may need to be above 50% to treat cardiomyopathy.^{22,23} However, it is important to stress that the therapeutic effect depends not only on the amount of dystrophin delivered but also on the advancement of the disease at the time of treatment. In older patients, who show profound loss of muscle fibers as well as marked fibrotic changes and fatty deposition, dystrophin delivery to muscle cells might have a limited therapeutic benefit.

Given the large mass requiring treatment in this disease, it is believed that the optimal mode of delivery may be *via* the vasculature. However, not all gene delivery systems are amenable to systemic delivery due to issues arising with the immune system and varying abilities to cross the blood/tissue barrier. Furthermore, it has become evident that DMD patients, who generally have an almost complete lack of dystrophin protein, may mount an immune response to the therapeutic gene, especially in those patients carrying deletions.²⁴ To overcome this concern, several groups are now investigating the potential of delivering utrophin, a dystrophin homologue, which is expected to be less immunogenic.^{25,26} This review focuses on the current methodologies for delivery of dystrophin to dystrophic muscles, however most methods discussed below can also be applied to the delivery of utrophin or other potential dystrophin surrogates.

One of the greatest challenges to DMD gene therapy resides in the size of the *dmd* gene, which at 2.2 Mb is one of the largest known genes. As previously mentioned, DMD arises from null mutations in the *dmd* gene that result in the near complete absence of dystrophin protein, or in rare cases from mutations that lead to production of a non-functional dystrophin, such as ones lacking critical domains near the carboxy-terminus of the protein.⁵ The allelic form, Becker muscular dystrophy (BMD), also arises from mutations in the *dmd* gene, however these mutations produce reduced amounts or truncated forms of dystrophin.^{27,28} The most promising viral vectors under investigation for gene therapy of DMD do not have large enough packaging capacities to carry a gene construct able to encode a full-length or known BMD-associated dystrophin protein. Therefore the focus has turned to development and characterization of internally deleted, mini- or micro-dystrophin constructs. Dystrophin is composed of 4 major structural domains: an N-terminal actin-binding domain; a central rod domain (a portion of which also binds actin) composed of 24 spectrin-like repeats and 4 hinge domains (the fourth carries a WW domain important for binding to part of the DGC); a cysteine-rich domain; followed by a distal C-terminal domain that interact with members of the DGC at the sarcolemmal membrane (Fig. 1A)²⁹. The idea that functional miniature dystrophin constructs may be useful came from the finding that BMD patients with very mild dystrophy can carry large deletions in the *dmd* gene.³⁰ The generation of transgenic *mdx* mice engineered to carry dystrophin constructs lacking varying domains revealed that the majority of the rod domain and the C-terminal domain are not essential for dystrophin function.^{31–39} Consequently, mini-dystrophin and highly miniaturized micro-dystrophin constructs are now being tested using both viral and non-viral modes of delivery to determine their potential use as therapeutic proteins (Fig. 1B).

VIRAL VECTORS FOR GENE THERAPY

Lentivirus

Murine retroviral vectors were among the first viral vectors to be tested for gene replacement therapy for DMD. Unfortunately, as these vectors were only capable of transducing proliferating myoblasts, the direct injection of a murine retrovirus carrying a mini-dystrophin construct into an *mdx* mouse resulted in poor transduction, with only a small percentage of fibers at the injection site expressing the construct.⁴⁰ Subsequently, newer retroviral vectors have been generated based on the human or feline lentiviruses. The lentivirus is a medium-sized enveloped virus containing an RNA genome of approximately 8 kb that, unlike early retroviral vectors, can efficiently transduce non-cycling cells including post-mitotic neurons, hepatocytes, and muscle fibers.^{41,42} Injection of a lentiviral vector expressing green fluorescent protein (GFP) into muscle tissue resulted in stable gene expression of half the muscle fibers at the injection site for at least 2 months and did not appear to elicit an immune response.⁴¹ More recently, lentiviral delivery of a dystrophin-GFP fusion protein was demonstrated to successfully transduce both skeletal muscle and resident satellite cells when injected into neonatal mice.⁴³ However, there remains a possibility that lentiviral vectors may induce an immune response when utilized at high doses, such as those that would be required for transduction in humans.⁴⁴ The key feature of lentiviral vectors is their ability to integrate into the host genome, a feature particularly desirable in the context of transduction of muscle progenitor cells as demonstrated by Kimura *et al.*⁴³ While not observed in this study, this feature is not necessarily desirable *in vivo* due to the risk of insertional mutagenesis. However, genetic correction and delivery of lentiviral-transduced satellite cells provides an attractive therapeutic option.

Adenovirus

A second type of viral vector for gene transfer is based on the adenovirus. The adenoviruses are medium sized, non-enveloped, icosahedral viruses that contain a double-stranded DNA genome. The wild type adenovirus has a genome of approximately 35 kb, which primarily contains 4 early transcription units (E1, E2, E3, E4) that have regulatory functions, and a late transcript that codes for structural proteins.^{45,46} Transcription of the adenovirus can be divided into early and late stages, which are separated by DNA replication. Adenoviral vectors efficiently transduce both dividing and non-dividing cells, which makes them attractive candidates for the transduction of skeletal muscle.⁴⁷ Furthermore, adenoviral infection with the most common serotypes is generally minimally pathogenic in the human population, and therefore adenoviral vectors can be considered relatively safe in this context.

Conventional adenoviral vectors for use in gene therapy are constructed by replacing the early viral genes with an exogenous expression cassette and are generated in packaging cell lines that express the early viral genes. First and second-generation adenoviral vectors lack varying combinations of the early viral genes, resulting in a transgene carrying capacity of approximately 8 kb. One of these first-generation adenoviruses was the first viral vector to successfully deliver a miniaturized human dystrophin cDNA to *mdx* mice via intramuscular injection.⁴⁸ However, first and second-generation adenoviral vectors tend to have leaky expression of the remaining viral proteins *in vivo*, which elicit a host immune response. This

immune response eliminates gene expression from transduced tissues and can lead to muscle damage and exacerbation of weakness.^{45, 49–57} Furthermore, the immunogenicity of these vectors precludes them from use in systemic administration due to the danger of producing a life-threatening systemic immune response.

The limited packaging capacity and immunogenicity of the conventional adenoviral vectors has been partially overcome by the generation of helper-dependent or ‘guttated’ adenoviral vectors. Guttated adenoviral vectors contain *cis*-acting DNA sequences that direct adenoviral replication and packaging but lack viral coding sequences.^{58–60} The use of these vectors has led to a reduced immune response, improved transgene expression, and a larger transgene carrying capacity.^{61–63} To this end, delivery of full length dystrophin to adult and neonatal *mdx* mice using guttated adenoviral vectors results in long-term expression and amelioration of the physiological and pathological symptoms of muscle disease.^{62,64,65} Despite these improvements in adenoviral vectorology, neutralizing antibodies arise against the vector after the first administration, thereby preventing repetitive administration⁶⁶, which would be required for persistent, long term transduction in humans. Also, delivery of very high doses of adenoviral vectors can result in induction of innate and cellular immune responses that are potentially lethal.^{67,68} Therefore, while adenoviral vectors are capable of delivering full-length dystrophin, resulting in efficient and moderately long-term transduction, improvements are required for these vectors to be utilized safely in gene therapy trials that require disseminated gene transfer.

Adeno-associated virus

Other viral vectors currently in use for DMD gene therapy are based on various serotypes of adeno-associated virus (AAV). Several AAV serotypes have a natural tropism for skeletal muscle.⁶⁹ Many AAV vectors efficiently transduce skeletal muscle fibers and the resulting gene expression can persist for years in healthy muscle, making these vectors of great interest for muscle gene therapy.^{65,70,71} AAV is a small, non-enveloped, non-pathogenic parvovirus containing an approximately 4.7 kb single stranded DNA genome which requires the presence of a helper virus for efficient replication and virus production.⁷² It was originally identified in 1965 as a contaminant in adenoviral preparations.^{68,73,74} The AAV capsid is composed of 3 proteins, VP1, VP2, and VP3, which are encoded by the Cap open reading frame (ORF). AAV also contains a Rep ORF, which encodes proteins involved in viral replication. Together the Cap and Rep ORFs span most of the AAV genome. The remainder of the genome is composed of 2 inverted terminal repeats (ITRs), which regulate packaging, genome stability, and DNA replication.

AAV has a biphasic life cycle, enabling it to either enter latency or produce an infection. AAV enters a host cell via interactions with both primary and co-receptors.⁷⁵ For AAV2, the primary receptor interaction occurs with the heparan sulfate proteoglycans (HSPG), and the co-receptors are fibroblast growth factor receptor 1 (FGFR1) and $\alpha v\beta 5$ integrin.^{76–78} Receptor binding allows viral endocytosis, after which the AAV particles are released from the endosome.⁶⁹ As AAV is a helper-dependent virus, it requires the presence of a helper adenovirus or herpes virus at this stage in order to undergo DNA replication and produce infective virions. In the absence of a helper virus, AAV can persist in a latent state. The

virus has presumably evolved this mechanism as a means of ensuring its persistence in the host.

AAV has been detected in many species, including nonhuman primates, canines, fowl, and humans.⁷⁹ To date, at least 11 strains of AAV have been identified from primates, and more than 100 sequences representing novel AAV clades have been reported.^{80–83} Recombinant AAV (rAAV) vectors, which are generated by placing the AAV ITR sequences on both sides of an expression cassette, are deleted of all viral genes. The ability to grow AAV vectors and use them for cell transduction of a protein, such as dystrophin, requires a transfer plasmid containing flanking ITRs, a promoter sequence, the transgene, and a polyadenylation signal. Generation of a recombinant AAV vector is accomplished by co-transfection of this transfer plasmid into a packaging cell line together with a second plasmid that contains the necessary adenoviral genes for replication and the Rep/Cap ORFs.^{84,85} Typically, rAAV vectors contain AAV2 ITRs and Rep genes, together with Cap ORFs from the serotype chosen for optimal tissue tropism. The ITRs provide an origin of replication and a packaging signal. Upon transduction, rAAV vectors support stable, long-term gene expression in muscle cells.^{86,32,87–90}

While rAAV vectors appear to be good candidates as gene therapy tools for DMD, the limited packaging capacity of the vectors remains problematic. Three different strategies have been applied to overcome this limitation. The first includes the use of miniaturized dystrophin genes, which as discussed below have proven to be quite successful in restoring muscle function in the mouse and dog models. The other 2 strategies involve trans-splicing and homologous based approaches. Trans-splicing involves the utilization of 2 or more rAAV vectors that have the transgene split between them. The 5' portion contains a 3' splice donor sequence, and the 3' portion contains a 5' splice acceptor site, and expression of the larger spliced mRNA occurs in co-transfected cells.³⁵ The recombination approach is similar in that 2 rAAV vectors that contain overlapping fragments of a larger gene are delivered simultaneously, resulting in homologous recombination at the overlap site to reconstitute the larger gene.^{91–93} These methods potentially enable the rAAV-mediated delivery of full-length dystrophin.

As discussed earlier, the key to effective gene therapy for muscular dystrophy is likely to be body wide distribution of the therapeutic gene, which will most likely require systemic delivery. While this has not proved possible by administration using adenoviral or lentiviral vectors, the ability to generate high titers and the natural tropism for musculature make rAAV vectors an attractive candidate. Whilst initial efforts to target the diaphragm and heart utilized invasive surgical procedures and required cofactors to induce vascular permeability that have the potential to induce toxicity,⁹⁴ the ability to effectively transduce both cardiac and skeletal muscle in the *mdx* mouse was demonstrated by single low pressure intravenous administration of rAAV6.^{95,32} rAAV6 efficiently transduced diaphragm and intercostal muscles following intravenous administration, demonstrating the ability of this vector to target all muscle groups required for DMD gene therapy.³² Intravenous or intra-articular injection of rAAV8 and rAAV9 also results in widespread transduction of the heart and skeletal musculature in both neonatal and adult mice.^{96,97} This has also been observed for

rAAV1,^{98,99} however rAAV6, 8, and 9 appear to transduce more effectively than rAAV1 when compared side-by-side by regional intravascular delivery.^{100,101}

Systemic transduction with rAAV1, 6, 8, and 9 appears to be possible in the absence of an immune response in mice. However intramuscular injection in the dog resulted in a robust T-cell mediated immune response to the viral capsid, yet it is important to note that this immune response could be avoided by transient immunosuppression.^{102–104} The chimeric rAAV2.5 capsid has been engineered based on the AAV2 capsid but contains 5 mutations based on rAAV1.¹⁰⁵ This vector combines the effective muscle transduction of rAAV1 with the heparin receptor binding ability of rAAV2 and has reduced antigenic cross-reactivity with both rAAV1 and rAAV2 serotypes in human clinical trials.¹⁰⁶ Human clinical trials using rAAV 1 and 2 have also led to the development of T-cell mediated immune responses against the viral capsids.^{107,106} It will be critically important to assess whether transient immunosuppressive strategies, such as those pioneered in the canine model of DMD, will effectively block these responses in patients.^{103,104} Perhaps of greater concern is the recent observation that some patients may elicit an immune response to the dystrophin protein itself, as was demonstrated in an AAV clinical trial.²⁴ This raises the possibility that some patients may not be amenable to the reintroduction of dystrophin by either viral or non-viral mediated strategies, and personalized gene therapy may be required based on the individual's mutation.

NON-VIRAL GENE THERAPIES

In addition to viral gene therapy, several non-viral replacement and repair approaches have been pursued for treatment of DMD. The replacement approach relies on delivery of a dystrophin cDNA by unencapsidated plasmids, while the repair approaches involve injection of antisense oligonucleotides (AONs) to generate an in-frame transcript by induced skipping of mutated exons, injection of chimeric oligonucleotides (RDOs) or oligodeoxynucleotides (ODNs) to correct single base mutations, and administration of drugs orally to suppress nonsense mutations by translational read through. These approaches are discussed below and compared in Table 1.

Delivery of unencapsidated plasmids

In the early nineties Wolff and others reported expression of reporter genes in muscles injected with 'naked' plasmid DNA.¹⁰⁸ Despite the initial low efficacy of the gene transfer (~1% of fibers expressing a reporter gene), simplicity, safety, and the low cost of the method raised widespread interest, and the study was followed by numerous investigations that focused on its optimization. Some of the improvements have involved sodium phosphate and DNase inhibitors protecting injected DNA from degradation,^{109,110} non-ionic carriers increasing plasmid diffusion through the tissue,¹¹¹ ultrasound,¹¹² and electroporation¹¹³ inducing cell membrane permeabilization. To date, electroporation in conjunction with pretreatment of muscle with hyaluronidase, which breaks down components of the extracellular matrix, have yielded the highest transfection efficiencies, comparable to those achieved with viral vectors.^{114–116} This method was used to transfer micro and full-length dystrophins into *mdx* muscle.^{117,115} Interestingly, the number of successfully transfected

fibers was markedly lower in the case of full-length dystrophin DNA when compared with microdystrophin plasmids, suggesting less efficient transfer of larger constructs.¹¹⁵

Although intramuscular injection of plasmids in combination with adjunctive electroporation leads to relatively high transfection efficiencies (~50% fibers expressing a reporter gene),^{114,116} the regional and systemic applications are clearly limited by its invasive character. Therefore, in parallel with local intramuscular injections of naked DNA, studies focusing on intravascular delivery of plasmids have been conducted. Liu *et al.* reported that delivery of plasmids containing full-length dystrophin cDNA into the tail vein, followed by surgical clamping of the effluent blood vessel of the diaphragm, leads to the presence of approximately 40% of dystrophin positive fibers in the diaphragm of the *mdx* mouse.¹¹⁸ In addition, intravascular administration of therapeutic DNA into dystrophic mice, in which aorta and vena cava were clamped just below the kidneys, yielded more than 30% positive myofibers throughout all the muscles of both legs.¹¹⁹

Regional delivery of naked DNA under high hydrostatic pressure can be easily scaled up and was successfully applied to large animal models.^{7,120–123} It is also important to stress that, while challenging in the case of viral vectors, repeated injections of plasmids are well tolerated and lead to a cumulative effect.¹²¹ These encouraging results prompted the first clinical trial,¹²⁴ where a group of 9 Duchenne and Becker patients underwent injection into the radialis muscle with a full-length dystrophin plasmid construct. Although the injected doses were low, a moderate dystrophin expression pattern was found in 6 patients. Importantly, no adverse effects of the treatment were observed. Furthermore, there are plans to deliver full-length dystrophin into arms of DMD patients using hydrodynamic limb vein administration, where the plasmid delivery is assisted by blocking the blood flow with a tourniquet and using very high venous pressure.^{8,122,125}

Although the number of dystrophin positive fibers is stable for at least a year following intra-arterial delivery into *mdx* mice, the number of plasmid DNA molecules decreases progressively over time.¹¹⁵ Hence, Bertoni, *et al.* investigated the effects of co-administration of dystrophin cDNA and a phage integrase,¹²⁶ which can mediate the integration of suitable plasmids into mammalian genomes. The study showed sustained higher levels of dystrophin expression in the case of plasmid integration; however, the risk of insertional mutagenesis with currently used integrases could limit the application of this approach.¹²⁷

Changing the mRNA splicing

DMD patients, as well as dystrophic animal models, display a small number of dystrophin-positive fibers, so-called revertant fibers.^{128–130} Although this phenomenon is enigmatic, revertant fibers are thought to be a result of splicing alterations in muscle precursor cells, which in turn leads to the restoration of the open reading frame and expression of truncated dystrophin in myofibers.¹³¹ This finding, in combination with the observation that BMD patients carrying in-frame deletions are often only mildly affected and that miniaturized dystrophins have been shown to be highly functional in mice,³¹ prompted the use of AON-based strategies to induce skipping of mutated exons to enable translation of a partially functional dystrophin protein.

AONs are used to block splice sites or splicing regulatory regions (enhancers) of pre-mRNA.¹³² Initial studies showed dystrophin restoration in cultured muscle cells from the *mdx* mouse^{133,134} and DMD patients^{135,136} following delivery of 2'-O-methyl phosphorothioate antisense oligonucleotides (2MeAONs), which are resistant to endonucleases and RNaseH and bind to RNA with high affinity.¹³² Injections of 2MeAONs into the *mdx* mouse, both intramuscularly^{137,138} and intravenously¹³⁹, have also been successful. Particularly, the studies carried out by Lu, *et al.* showed that combined systemic delivery of 2MeAONs and non-ionic block copolymers yields dystrophin expression in all skeletal muscles (up to 5% of normal levels).¹³⁹

Much higher levels of dystrophin expression were obtained more recently with alternative compounds to 2MeAONs, peptide nucleic acids (PNAs)^{140,141} and phosphorodiamidate morpholino oligonucleotides (PMOs).¹⁴² Particularly, in the latter study, 7 weekly intravascular injections of PMOs resulted in 10–50% of normal dystrophin levels in skeletal muscles. However, systemic delivery of 2MeAONs and PMOs failed to induce exon skipping in the heart, and thus their use would exacerbate the existing cardiomyopathy in DMD.¹⁷ This problem was first addressed by inducing exon skipping via administration of an AAV vector expressing antisense sequences linked to a modified U7 small nuclear RNA gene,^{143,99} and more recently by administration of a conjugate of PMOs and cell-penetrating peptides (PPMOs).^{134,144–148} PPMO treatment is particularly effective, as it leads to almost full restoration of dystrophin in cardiac and all skeletal muscles. Although promising, caution has to be taken, as recent preclinical studies showed that the PPMO targeting exon 50 (AVI-5038) causes toxicity in cynomolgus monkeys at doses that were found to be safe in mice.¹⁴⁹

One of the hurdles facing successful AON-based therapy is a mutation-based customized approach. Theoretically, exon-skipping could be applied to 90% of patients, and 16% of them would benefit from targeting exon 51.¹³² Using AON cocktails could expand the number of patients profiting from a single treatment. Such strategy was successfully employed in dystrophic dogs, in which multiexon skipping resulted in therapeutic expression of dystrophin throughout the body.¹⁵⁰ Furthermore, the AON treatment has only a transient beneficial effect and requires repeated administrations, e.g. at biweekly intervals.¹⁵¹ Nonetheless, the results of initial clinical trials have been very encouraging,^{135,152–154} as biopsies taken from patients injected with PRO051 2MeAONs¹⁵² and AVI-4658 PMOs^{153,154} revealed dystrophin expression without apparent adverse effects. Future studies should address the long-term toxicological and immunological consequences of repeated, lifelong delivery of AONs.

Genome-editing strategies

Single mutations in the dystrophin gene can be repaired by application of RDOs or ODNs, which induce endogenous DNA repair mechanisms.¹⁵⁵ RDOs and ODNs carrying a non-mutated base generate a mismatch with a single mutant base upon binding to complementary genomic DNA. This induces the DNA repair machinery and replacement of the mutated base with the correct one. As the *mdx* mouse and *cxmd* dog carry single point mutations that result in premature termination of the dystrophin reading frame,^{49,156,157} the genome

editing-based therapy was applied to these animal models. Following intramuscular injections of RDOs and ODNs, permanent correction of the mutations was observed, however in only a small number of fibers.^{158–160} Utilizing ODNs made of PNA bases resulted in improved correction efficiencies in a more recent study, in which dystrophin expression was observed in up to 250 fibers at the injection site.¹⁶¹

Ribosomal read through of premature stop codons

Approximately 10% of DMD patients carry nonsense mutations that could be treated with chemicals that induce read through of premature stop codons.¹⁶² Gentamicin treatment of the *mdx* mouse led to slightly increased dystrophin expression.¹⁶³ Although the study seemed encouraging, few labs were able to repeat the results, and the majority of subsequent human trials failed to show any therapeutic benefit from this method.^{164,165} In the most recent study, 3 out of 12 DMD patients treated with gentamicin for 6 months revealed 13 to 15% of normal dystrophin levels.¹⁶⁶ However, the remaining patients revealed no or only moderate dystrophin expression. As an alternative, an orally bioavailable drug called ataluren (formerly known as PTC124) was developed and tested by PTC Therapeutics.^{167,168,162} The results showed dystrophin restoration to ~20% of normal levels and partially improved contractile properties of treated skeletal muscles for a limited period of time in *mdx* mice¹⁶⁷. Unfortunately, no convincing evidence has been presented that ataluren induces significant dystrophin expression in cardiac muscle. As with gentamicin, ataluren treatment also failed to provide any therapeutic benefit to DMD patients in human trials.^{168,162}

CELL-MEDIATED THERAPIES FOR DMD

Cell therapies, in which cells bearing a functional dystrophin gene are transplanted to treat muscular dystrophy, have been gaining increased attention in recent years. Potentially therapeutic cells can be obtained either from a patient, in which case cells are corrected *ex vivo* and re-implanted (autologous transfer), or from an unaffected donor and injected into a dystrophic patient (allogeneic transfer). Ideally, transplanted cells should be able to migrate from blood to muscle and should not only form myotubes but also enter the satellite cell niche and self-renew to provide a long-lasting treatment.

Myoblast and satellite cell transplantation

The satellite cell is the principal skeletal muscle stem cell that is defined by expression of transcription factor Pax7 and localization on myofibers beneath the basal lamina (Fig. 2).^{169,170} In healthy muscle, satellite cells remain in a quiescent state and make up approximately 2–7% of the nuclei associated with fibers.¹⁶⁹ In response to injury, satellite cells become activated and differentiate into myoblasts, which proliferate and fuse to repair or replace the damaged fibers. A fraction of activated satellite cells returns to quiescence to maintain the pool of progenitor cells.

Due to the ease of isolation and culturing *in vitro*, myoblasts were the initial choice for cell-based therapies to treat muscular dystrophy. Experiments carried out in the late 1980s by Partridge *et al.* showed that intramuscular injections of normal myoblasts into the *mdx*

mouse result in cell fusion and expression of dystrophin in *mdx* myofibers.¹⁷¹ Subsequent clinical trials, however, failed to deliver significant levels of dystrophin.^{172–174} Efficiency of myoblast engraftment in DMD muscles can be improved by combining multiple injections of high numbers of myoblasts with tacrolimus-based immunosuppression. This approach resulted in normal dystrophin production in approximately 10% of fibers at injection sites.^{161,175–177} Although transplanted myoblasts contribute to the satellite cell pool,^{178–180} their poor survival and limited migration *in vivo* prompted the search for alternative cell types for cell-based therapies.

A pure population of satellite cells was first obtained from a *Pax3*-GFP knock-in mouse by fluorescent activated cell sorting (FACS).¹⁸¹ When delivered into the *mdx* mouse, purified satellite cells engrafted within the muscle and contributed to the satellite cell pool at much higher efficiencies than myoblasts. In recent experiments, FACS was employed to isolate a highly proliferative subset of satellite cells from non-transgenic mice based on the expression of various satellite cell-surface markers.^{182,183} Upon administration, these cells contributed to up to 94% of *mdx* fibers markedly improving their contractile functions.¹⁸² Furthermore, they re-engrafted the satellite cell niche and contributed to fiber regeneration in response to serial tissue damage.¹⁸³

Although the transfer of satellite cells restores dystrophin expression to higher levels when compared to the myoblast-based approaches, the methods are equally hindered by the inability of both cell types to migrate over long distances. As such, their application is currently limited to local injections. Similarly restraining is the reduced myogenic potential of cultured satellite cells. This is especially important when considering the autologous cell transplant that requires genetic manipulations.¹⁷⁰ The promising study by Gilbert *et al.* showed, however, that this problem might be solved by propagating satellite cells on matrices that mimic the rigidity of muscle tissue.¹⁸⁴

Systemic delivery of stem cells

In addition to satellite cells, several other stem cell types possess the ability to differentiate into myofibers. These include total bone marrow-derived stem cells, blood- and marrow-derived side population and CD133+ cells, mesenchymal cells, and mesoangioblasts. In contrast to myoblasts and satellite cells, all of these have been shown to be compatible with systemic delivery through the circulatory system.

Bone marrow- and muscle-derived stem cells

Ferrari, *et al.* reported the first potential therapeutic applications of bone marrow-derived cells for muscular dystrophy, as following systemic delivery of fractionated total bone marrow cells, they observed their incorporation into regenerating muscle fibers.¹⁸⁵ Subsequent studies determined that a Hoechst 33342-stained subpopulation of bone marrow cells, called side population (SP) cells,^{186,187} efficiently incorporated into *mdx* muscles upon administration into the blood stream, composing up to 4% of dystrophin-positive fibers.¹⁷² A similar pattern of dystrophin expression was observed after administration of SP cells isolated from muscle; however, in contrast to bone marrow-derived SP cells, the muscle-derived cells were additionally detected at the satellite cell position. More recently, *ex vivo*

corrected autologous muscle SP cells bearing microdystrophin were delivered into the blood stream of dystrophic mice.^{188,189} The amount of dystrophin produced was, however, below therapeutic levels. Dezawa, *et al.* obtained much higher numbers of dystrophin-positive fibers (20–30% of centrally nucleated fibers) in the *mdx* mouse upon administration of *in vitro* engineered mesenchymal cells.¹⁹⁰ Importantly, these cells also persisted as satellite cells and were able to contribute to regeneration following muscle injury, proving their future potential as a therapeutic tool.

The CD133+ cell is another progenitor cell type capable of differentiating into muscle.^{191,192} In a recent study, CD133+ stem cells were isolated from DMD patients, corrected *ex vivo* using a lentiviral vector and transplanted into *mdx* mice. Intra-arterial delivery resulted in significant dystrophin expression in limb muscles that was paralleled by improvement of muscle morphology and function. Autologous transplantation was also tested for safety in a phase I clinical trial.¹⁹³ Neither local nor systemic side effects were found after injection of CD133+ cells into abductor digiti minimi muscle, which paves the way for future clinical trials with gene corrected CD133+ cells.

Mesoangioblasts

Transplantation of blood vessel-associated stem cells, called mesoangioblasts, also ameliorates the dystrophic phenotype.^{194,195} Systemic delivery of heterologous and autologous mesoangioblasts transduced with a lentiviral vector expressing human microdystrophin gave particularly remarkable results in the *cxmd* dog. The treatment resulted in dystrophin expression in 5–70% fibers and general improvement of the condition of the injected dogs. Despite the controversy that followed the study,^{196–198} mesoangioblasts represent one of the most promising cell-based therapies for muscle.¹⁹⁹

Recently, Tedesco *et al.* presented a relatively new approach to deliver dystrophin into dystrophic muscles.¹⁹⁹ A human artificial chromosome carrying the whole dystrophin gene was transferred into mesoangioblasts isolated from *mdx* mice. When these cells were injected *via* intramuscular or intra-arterial approaches into *mdx* mice, markedly enhanced motor capacity was observed despite moderate dystrophin expression of 25% and 13.5%, respectively. Most importantly, the artificial chromosome was stable for 30 population doublings and 8 months *in vivo*, and at least some of the transplanted mesoangioblasts self-renewed and localized to the satellite cell pool.

Generating myoblasts from embryonic stem (ES) cells

While various cell types isolated from adult muscle can exhibit regenerative potential, an ES cell-based therapeutic approach has significant advantages, as it results in a more primitive cell with a higher replication potential to enable more durable engraftment.²⁰⁰ ES cells are a pluripotent cell population derived from the inner cell mass of the pre-implantation blastocyst. ES cell lines have been derived from mouse and human blastocysts and can be directed to differentiate into all 3 germ layers of the embryo containing mesendoderm, definitive endoderm, visceral endoderm, mesoderm, and neuroectoderm.^{201–203} As a result, the successful isolation of human ES cells raised the hope that they may provide a universal

tissue source for the treatment of many human diseases. However, differentiation of both human and mouse ES cells into specific cell types presents a challenge.

The most primitive protocol for ES cell differentiation involves formation of embryoid bodies, which involves directing ES cells to differentiate by culturing in defined media. However, this protocol yields few mesenchymal progenitors. Mesenchymal progenitors can be derived from human ES cells that are over-expressing either *IGFII* or *Pax3*.^{204–206} Culture of human ES cells on a monolayer of the murine stromal OP9 cells, which induces blood cell differentiation in mouse ES cells, also induces the formation of mesenchymal stem cells, which can be directed to form myotubes *in vitro*.^{207–209} This method has subsequently been performed in the absence of the stromal layer by supplementation with various cytokines.²¹⁰ This result demonstrates that human ES cells can generate paraxial mesodermal progenitor cells without gene manipulation, which provides a key step forward in these protocols. Recently, Darabi, *et al.* have extended this work and demonstrated that myogenic progenitors can be derived from ES and induced pluripotent stem (iPS) cells (see below) by conditional expression of Pax7.²¹¹ Transplantation of these progenitors into muscles of *mdx* mice led to widespread regions of dystrophin-positive myofibers.

While stem cell therapies have potential as effective therapies for the muscular dystrophies, transplanted cells would have to be generated from existing human ES cell lines and are therefore likely to induce a host rejection response. The advance of iPS cell therapy may overcome this problem. The term iPS cell defines a population of somatic cells that have been reprogrammed *in vitro* to a pluripotent state.²¹² To date, this has been achieved for both adult and embryonic fibroblasts, murine liver and stomach cells, pancreatic beta cells, lymphocytes, and neural progenitor cells.^{213–215,127,216–219,212,220,221}

iPS cells are generated by ectopic expression of Oct4 and Sox2, in combination with either Klf4 and c-Myc or Lin28 and Nanog in somatic cells.^{127,216,217,219,212,220,221} Chimeras generated from iPS cells generated with the c-Myc transgene demonstrate increased tumorigenicity.²¹⁶ Consequently, current protocols do not utilize c-Myc expression and instead use the Lin28/Nanog combination or replace c-Myc with n-Myc which is less tumorigenic.²²² The simultaneous over-expression of this gene combination induces transcription of the endogenous pluripotent transcriptional machinery in somatic cells, causing the cells to adopt an ES cell-like morphology. iPS cells are morphologically indistinguishable from ES cells, can be maintained in culture under the same conditions as ES cells, and form teratomas containing tissue types representative of all 3 embryonic gene layers when injected into mice.^{219,212} This suggests that any protocol that induces mesenchymal differentiation of ES cells should also do so in iPS cells, and therefore patient-derived cells can be reintroduced as therapeutic cells, overcoming the potential host immune response posed by ES cells.

Generating myoblasts from somatic cells

Although the generation of myoblasts from patient-derived iPS cells is a huge advance in cell-mediated therapy for DMD, it was recently demonstrated that fibroblasts can be induced to form transplantable myoblasts without the requirement of first becoming a pluripotent cell. Even at a young age, the muscles of DMD patients have fibrotic changes and fatty

deposition that is believed to indicate that muscle repair may be overtaxed.⁹ The presence of these fibrotic changes suggests that there are many fibroblasts present in the muscle fibers of DMD boys. Overexpression of the myogenic factor *MyoD* in cultured fibroblasts induces myogenic conversion of these cells.^{223–225} These myoblasts fuse with existing muscle fibers upon transplantation into a healthy mouse muscle.^{223–225} For this to be a viable therapy for DMD, each muscle would have to be treated individually as the transplanted cells can only be delivered by intramuscular injection. However, one particularly exciting potential for this approach is use of a systemic gene delivery method to transduce fibroblasts for myogenic conversion *in vivo*. While this is an exciting theory, to date it is not possible to selectively transduce a specific population of cells *in vivo*. Therefore, although conversion of invading fibroblasts to myoblasts *in vivo* is an attractive approach, this methodology is many years away in terms of a therapeutic strategy.

CONCLUSIONS

Even though there is still no cure for DMD and current treatment options are limited to physiotherapy and corticosteroids,^{226,227} considerable progress has been made in the last 2 decades to develop a number of potential therapeutic interventions. AAV vector- and PPMO-based methods are particularly promising, as they have been shown to restore dystrophin expression not only in skeletal muscles but also in the heart. Scientists have also managed to overcome intrinsic limitations of therapies by developing synergistic approaches. Of these, autologous transplantation of *ex vivo* corrected stem cells with lentiviral vectors seems to have the greatest potential.

In addition to the therapies described here, a number of other interventions are being developed. These include approaches to pharmacologically increase utrophin levels^{189,228,21} and to modulate expression of signaling molecules having an effect on muscle strength, regeneration and/or mass.^{222,229–232} As DMD is a heterogeneous disease with a variety of different types of mutations spanning the entire dystrophin gene, some treatments might be more suitable for particular patients. A wide range of available therapies in the future would enable a personal therapeutic design, based on the applicability, cost and potential risk of a particular method.

In conclusion, the described therapies have moved from local to systemic applications and are beginning to be scaled up to meet the human therapy needs. Given the satisfactory results from cell- and animal-based studies, many of the therapies have gone through or are being assessed in early-stage clinical trials. The described safety of some of them suggests we are coming closer to finding an effective treatment for many types of muscular dystrophy.

Abbreviations

DMD	Duchenne muscular dystrophy
<i>mdx</i>	mouse model for DMD
<i>cxmd</i>	canine model for DMD

DGC	Dystrophin-Glycoprotein Complex
BMD	Becker muscular dystrophy
AAV	adeno-associated virus
GFP	green fluorescent protein
ORF	open reading frame
ITR	inverted terminal repeats
AON	anti-sense oligonucleotide
RDO	chimeric oligonucleotide
ODN	oligodeoxynucleotide
2MeAON	2'-O-methyl phosphorothioate antisense oligonucleotide
PNA	peptide nucleic acid
PMO	phosphorodiamidate morpholino oligonucleotide
PPMO	a PMO coupled to a cell-penetrating peptide
FACS	fluorescent activated cell sorting
SP	side-population (cell)
ES	embryonic stem (cell)
iPS	induced pluripotent stem (cell)

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Follistatin-mediated skeletal muscle hypertrophy is regulated by Smad3 and mTOR independently of myostatin. *Journal of Cell Biology*. 2012; 197:997–1008. [PubMed: 22711699]

FIGURE 1.

(A) Dystrophin is a member of a multimeric protein complex termed the dystrophin-glycoprotein complex (DGC), which serves to link the cytosolic actin skeleton of the muscle fiber to the extracellular matrix. The N-terminal and much of the central rod domain of dystrophin form a lateral interaction with actin filaments immediately below the sarcolemma. The dystrophin C-terminal region links to the DGC via an interaction with β-dystroglycan (β-Dg) through a dystroglycan-binding domain formed by the WW domain and a cysteine-rich (CR) domain. β-dystroglycan binds α-dystroglycan, which is an extracellular

protein that binds laminin. The C-terminal domain of dystrophin (CT) binds to members of the syntrophin (Syn) and dystrobrevin (Dbn) protein families. The syntrophins also bind to neuronal nitric oxide synthase (nNOS). The DGC also contains a sarcoglycan/sarcospan sub-complex that consists of α -, β -, γ -, and δ -sarcoglycan (Sg) and sarcospan (Spn). **(B)** Micro-dystrophin constructs that lack a large portion of the rod domain (R4–R23), and the CT domain are currently being developed and tested to treat DMD. These micro-dystrophins are highly functional and are capable of restoring the DGC as shown. The only differences in the DGC that associates with micro-dystrophin are a lack of nNOS binding and fewer associated syntrophins.

FIGURE 2.

EDL muscle cryosections from wt and *mdx* mice immunostained for laminin, Pax7 and a nuclear marker (DAPI). Pax7-positive nuclei lying beneath the basal lamina mark satellite cells (arrows). Arrowheads in the *mdx* section point to nuclei located in the center of fibers. Central nucleation is a hallmark of recent myofiber regeneration.

Table 1

A list of non-viral gene strategies and resulting outcomes. IM and IV stand for intramuscular and intravascular injections, respectively.

Method	Efficiency	Delivery	Comments
Unencapsulated DNA plasmids			
Plasmid DNA	Very low (1%)	Regional (IM)	
Plasmid DNA + electroporation + hyaluronidase	Moderate (50%)	Regional (IM)	Very invasive
Plasmid DNA + high pressure	Moderate (40%)	Regional (IV)	Moderately invasive
Antisense oligonucleotide (AON)-based exon skipping			
2MeAONs + non ionic block copolymers	Low (5%)	Systemic (IV)	Mutation-specific therapy
PMOs	Moderate (10–50%)	Systemic (IV)	Mutation-specific therapy
PPMOs	Moderate/High (25–100%)	Systemic (IV)	Mutation-specific therapy; target both skeletal muscles and heart
Oligonucleotide-mediated gene editing			
RDOsand ODNs	Low	Regional (IM)	Mutation-specific therapy; suitable only for single-base mutations
Ribosomal readthrough of premature stop codons			
PTC124	Moderate (20%)	Systemic (oral)	Mutation-specific therapy