

Review Article

Recent advances in innovative therapeutic approaches for Duchenne muscular dystrophy: from discovery to clinical trials

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Abstract: Duchenne muscular dystrophy (DMD) is an X-linked progressive degenerative muscle disorder caused by the absence of dystrophin. There is no curative therapy, although innovative therapeutic approaches have been aggressively investigated over recent years. Currently, the international clinical trial registry platform for this disease has been constructed and clinical trials for innovative therapeutic approaches are underway. Among these, exon skipping and read-through of nonsense mutations are in the most advanced stages, with exon skipping theoretically applicable to a larger number of patients. To date, exon skipping that targets exons 51, 44, 45, and 53 is being globally investigated including in USA, EU, and Japan. The latest announcement from Japan was made, demonstrating successful dystrophin production in muscles of patients with DMD after treating with exon 53 skipping antisense oligonucleotides (ASOs). However, the innovative therapeutic approaches have demonstrated limited efficacy. To address this issue in exon skipping, studies to unveil the mechanism underlying gymnotic delivery of ASO uptake in living cells have been conducted in an effort to improve *in vivo* delivery. Further, establishing the infrastructures to integrate multi-institutional clinical trials are needed to facilitate the development of successful therapies for DMD, which ultimately is applicable to other myopathies and neurodegenerative diseases, including spinal muscular atrophy and motor neuron diseases.

Keywords: Duchenne muscular dystrophy, dystrophin, exon skipping, drug delivery, clinical trial networks, outcome measures

Introduction

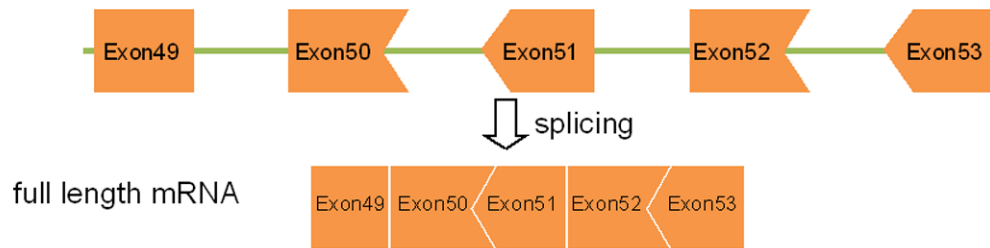
Duchenne muscular dystrophy (DMD) is an X-linked muscular disorder that is estimated to affect 1 in 3,800-6,000 live male births [1]. Affected individuals are usually asymptomatic or they may exhibit mildly delayed developmental milestones during infancy to early childhood with increased serum creatine kinase activities [2]. Most patients are diagnosed between 4 and 5 years of age [3], when they start to show signs of physical disability including walking difficulty. They then manifest progressive, systematic muscular weakness and usually become nonambulatory and wheelchair dependent before their teens [2]. Respiratory function decreases with advancing age and cardiomyopathy eventually develops, which is currently

the major cause of morbidity and mortality [4]. Without intervention, the mean age at death is around 19 years, but multidisciplinary care such as noninvasive mechanical ventilation significantly improves survival [2].

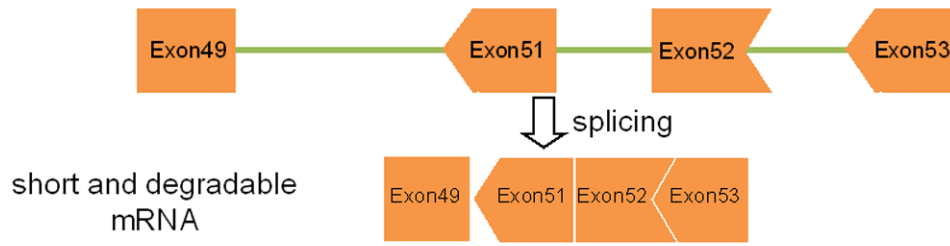
DMD is caused by a mutation in the *DMD* gene located on Xp21, and its protein product, dystrophin, forms the dystrophin-associated glycoprotein complex (DGC) at the sarcolemma, which links the muscle sarcomeric structure to the extracellular matrix [5] and protects the sarcolemma from contraction-induced injury [6]. The *DMD* gene encodes 79 exons, and the molecular weight of dystrophin is 427 kDa [7]. Dystrophin has four distinct functional domains, *i.e.*, the N-terminal actin-binding domain, central rod-like domain, cysteine-rich domain, and

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A Normal *DMD* gene transcript



B Exon 50 deletion



C Exon 51 skipping for exon 50 deletion

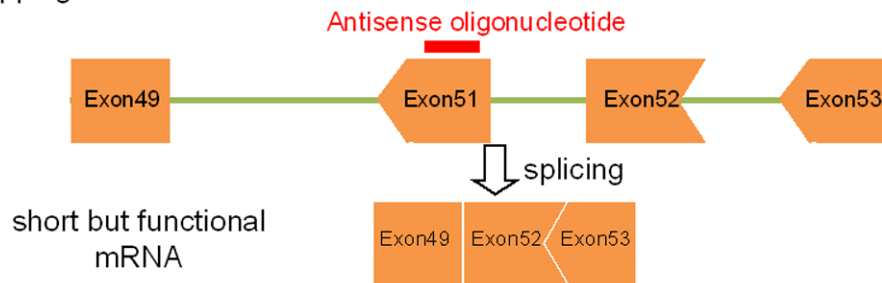


Figure 1. Schematic illustration of *DMD* gene transcripts. A. The normal *DMD* gene transcript that generates the mRNA for full length dystrophin. B. Patients with an out-of-frame, exon 50 deletion mutation generate the mRNA for short and degradable dystrophin. C. Exon 51 skipping in patients with an exon 50 deletion converts the out-of-frame transcript into an in-frame transcript, which can generate short but functional dystrophin.

C-terminal domain. Together with the rod domain, the cysteine-rich domain links dystrophin to the transmembrane DGC. In patients with DMD, the protein dystrophin is absent [7] and the muscle fibers become vulnerable to damage caused by mechanical stretching [8].

Mutations in the *DMD* gene are associated with two types of muscular dystrophy, DMD and Becker muscular dystrophy (BMD), depending on whether the translational reading frame is lost or maintained [9]. BMD is a clinically milder form of DMD, which is characterized by features similar to DMD, except that patients remain ambulant until at least age 16 years and display variable severity [10]. When the *DMD* mutation is “in-frame” and a semi-functional protein is produced, patients are likely to

develop BMD, whereas when the mutation is “out-of frame” patients undergo a disruption of the translational reading frame, which leads to the loss of dystrophin and development of DMD [11]. It should be noted that some patients do not conform with this reading-frame hypothesis, and the causes of these exceptions remain unclear. The most recent analyses of DMD mutations have demonstrated that 80% of all mutations are large mutations, which involve one exon or larger, among which 86% are deletions and 14% are duplications [9]. Small mutations, which affect segments smaller than one exon, contribute 20% of all mutations, wherein half are nonsense mutations [9]. For deletions and duplications, a nonrandom distribution of mutations has been identified in the *DMD* gene,

with two hot spots, *i.e.*, the distal (exons 45-55) and proximal (exons 2-20) hot spots [9].

Currently, there is no curative therapy for DMD and glucocorticoids are the only available medication that slow the decline of muscle strength and function [2]. However, several therapeutic strategies are now under aggressive investigation as well as clinical trials (<https://clinicaltrials.gov>), and guidance from the US Food and Drug Administration was recently announced to help accelerate the development of drugs for DMD (<http://www.fda.gov/>). At the same time, clinical trial network, an infrastructure to integrate multi-institutional clinical trials have been established for the neuromuscular field, as represented by the TREAT-NMD (<http://www.treat-nmd.eu/>). In Japan, the Registry of Muscular Dystrophy (Remudy) (<http://www.remudy.jp/english/>) has been established and runs national registries for muscular disorders including dystrophinopathy, in collaboration with the TREAT-NMD alliance. In addition, the Muscular Dystrophy Clinical Trial Network (MDCTN) (<http://www.mdctn.jp/>) has been established to create opportunities for clinical trials and accelerate clinical discoveries. Both Remudy and MDCTN, operated by the National Center of Neurology and Psychiatry, Tokyo, provide infrastructures to ensure that the most promising new therapies reach patients as quickly as possible in Japan.

Current therapeutic approaches to DMD can be categorized into two groups based on their strategic goals: therapies that aim to restore dystrophin expression and those that aim to compensate for the lack of dystrophin. Promising results have been reported from both therapeutic approaches. With significant recent advances in research for DMD therapy, we will review the current status of clinical trials centered on innovative therapies for DMD, as well as discuss the challenges and future perspectives. We will also describe the processes of discovery and underlying rationales for these therapeutic advances.

Therapies to restore dystrophin expression

Exon skipping

Exon skipping induced by antisense oligonucleotides (ASOs) modulates the dystrophin pre-mRNA splicing process, thereby restoring the

reading frame of the *DMD* gene (**Figure 1**) [10]. Thus, by generating a dystrophin protein that is shortened but functional instead of prematurely truncated and presumably degradable, in patients with DMD, exon skipping could convert the DMD phenotype into a milder BMD phenotype.

In the late 1980s, careful examination of muscle pathology led to the identification of rare dystrophin-positive fibers among otherwise dystrophin-negative fibers in DMD, which are now known as revertant fibers [12]. Although the biological mechanism responsible for generating revertant fibers is not yet fully understood, it has been reported that a variety of naturally occurring exon skipping events can bypass the original pathogenic mutation to produce revertant fibers [13]. In the beginning of the 1990s, Nicholson suggested a potential therapeutic advantage of exon skipping in DMD by extending the existing frame-shift deletion mutation to an in-frame mutation, thus rescuing dystrophin expression [14]. In addition, the use of ASOs as gene expression modulators was investigated *in vitro* and they were reported to be potentially beneficial in treating β -thalassemia by hybridizing with targeted RNA, suppressing aberrant splicing patterns to restore correct splicing [15]. Subsequent *in vitro* evidence demonstrated the modulation of dystrophin pre-mRNA splicing by an ASO [16], which facilitated the development of exon skipping therapy for patients with DMD.

Studies of exon skipping have shown that it is applicable to up to 83% of all DMD mutations [17]. Theoretically, patients who harbor exon deletions, duplications or small mutations (deletions, insertions, splice site mutations, and point mutations) could benefit from exon skipping. For deletions and small mutations, the skipping of one or two additional exons is usually adequate to restore the reading frame [17]. However, exon skipping is more challenging for duplications because ASOs cannot discriminate between the original and duplicated exons, which may induce the skipping of both or either exon, thereby yielding out-of-frame transcripts instead of the desired in-frame transcripts [17].

A mutation hotspot exists between exons 45 and 55, and the skipping of exon 51 is considered to be applicable to the largest subset of

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Table 1. Descriptions of antisense oligonucleotides used for exon skipping

Chemical	Description	Status	Ref.
2'OMePS	Adopts N-type sugar conformation. Increased binding RNA affinity and improves nuclease resistance.	Clinical trial	[10, 22]
PMO	Charge-neutral, nucleic acid bases are bound to morpholine moiety. Reduced off-target effects, increased water solubility, nuclease resistance, less immune response.	Clinical trial	[19, 20]
LNA	Sugar ring is locked by a O2'C4' methylene linkage adopting an RNA-like C3'-endo conformation. Exhibits unprecedented binding affinity to complementary DNA or RNA.	Pre-clinical	[10]
PNA	Charge-neutral, sugar backbone is replaced with the N-(2-aminoethyl)-glycine. Has high degree of nuclease and protease resistance and higher target-binding affinity.	Pre-clinical	[10, 29]
tcDNA	Possess additional three carbon atoms between C5' and C3'. Has increased RNA affinity, hydrophobicity, nuclease resistance. Spontaneously forms nanoparticles which could potentially improve cellular uptake.	Pre-clinical	[30]
PPMO, Pip-PMO, vPMO	New generation PMO derivatives containing cell-penetrating moieties with enhanced cell uptake.	Pre-clinical	[28]

2'OMePS, 2'-O-methyl-phosphorothioate oligonucleotide; PMO, phosphorodiamidate morpholino oligomer; LNA, locked nucleic acid; PNA, peptide nucleic acid; tcDNA, tricyclo DNA; PPMO, peptide-conjugated PMO; Pip-PMO, PMO internalization peptide-conjugated PMO; vPMO, vivo-morpholino.

patients. Therefore, ASOs that target exon 51 were the first to be developed clinically [18]. Several ASO chemicals have been investigated, among which the 2'-O-methyl-phosphorothioate oligonucleotide (2'OMePS) and phosphorodiamidate morpholino oligomer (PMO) are the major drug candidates currently under advanced phase evaluation in clinical trials (**Table 1**). Drisapersen developed by Prosensa (now BioMarin) employs a 2'OMePS chemistry in which the internucleotide phosphorothioate linkages are negatively charged; eteplirsen, a PMO-based drug developed by Sarepta Therapeutics, is a charge-neutral compound. The advantages of charge-neutral PMOs over 2'OMePS are reduced off-target effects, increased water solubility and nuclease resistance [19], as well as a stronger binding affinity for RNA [20]. Furthermore, PMOs do not elicit an immune response through Toll-like receptors and the interferon system [20]. Studies that compared 2'OMePS and PMOs concluded that skipping efficiencies are sequence-dependent and not chemistry-dependent, but PMOs may be slightly less sequence-specific than 2'OMePS [21]. The results of a clinical trial showed that drisapersen could not obtain significant improvements in its primary outcome measure, the 6-min walk test (6MWT), in both phase 2 and phase 3, double-blind, placebo-controlled clinical trials [22, 23]. Drisapersen is now being evaluated to determine its efficacy in younger patients aged less than 7 years [23]. An application for marketing approval for drisapersen was reviewed by FDA, however, in January 2016, FDA has concluded that the standard of substantial evidence of effectiveness is not met therefore the approval is not ready in its present form ([0309\). A phase 2, double-blind, placebo-controlled study of eteplirsen reported an increase in the percentage of dystrophin-positive fibers to 23% compared with the normal level, whereas the placebo-treated patients exhibited no increase \[24\]. An extended open-label study demonstrated stabilization of the ambulatory distance in 6MWT among those treated with eteplirsen compared with placebo/delayed treatment patients \[24\]. However, further evaluations using a larger number of participants, a wider dose range, and duration studies are necessary to obtain conclusive data. Recently an open label phase 3 clinical trial has started for eteplirsen \(NCT02255552\) and the FDA is reviewing of its new drug application on May 26, 2016.](http://investors.bmrn.com/releasedetail.cfm?ReleaseID=95-</p>
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Progress in developing ASOs targeting exons other than exon 51 is being made, and ASOs for exon 44, 45, and 53 skipping are in the early phase of clinical trials (**Table 2**). The latest announcement for exon 53 skipping has been made by Japan, demonstrating the restoration of dystrophin mRNA in low- (1.25 mg/kg), medium- (5.0 mg/kg), and high-dose (20 mg/kg) groups after the treatment with PMO. Moreover, the high-dose group showed dystrophin protein recovery. There were no serious adverse events, although anemia and a slight effect on renal function were reported. Preparation for the next-phase 1/2 clinical trials is now being made for this exon 53-skipping ASO (<http://www.nippon-shinyaku.co.jp/english/news/?id=2556>).

Future approaches to exon skipping therapies include multi-exon skipping and enhancement of drug delivery for increasing skipping efficiency. Multi-exon skipping of DMD exon 45-55 is

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Table 2. Descriptions of registered clinical trials for studies that aim to restore dystrophin (Current status, December 2015)

Drug/compound	Description	Company/Institute	Status	Clinical trial no.	Remarks	Ref.
Exon skipping						
Drisapersen	2'OMePS targeting exon 51	GlaxoSmithKline	Phase 3 completed	NCT01254019	Studies targeting patients older than 5 years. No statistically significant improvement in 6MWT.	[23]
Drisapersen	2'OMePS targeting exon 51	BioMarin Pharmaceutical	Phase 3	NCT01803412	Extension studies on patients who previously have received drisapersen.	
Drisapersen	2'OMePS targeting exon 51	BioMarin Pharmaceutical	Phase 1/2	NCT01910649	Assessing IV dosing as an alternative route of administration which previous administrations were done via SC.	
PRO044	2'OMePS targeting exon 44	BioMarin Pharmaceutical	Phase 2	NCT02329769		
PRO045	2'OMePS targeting exon 45	BioMarin Pharmaceutical	Phase 2b	NCT01826474		
PRO053	2'OMePS targeting exon 53	BioMarin Pharmaceutical	Phase 1/2	NCT01957059		
Eteplirsen	PMO targeting exon 51	Sarepta Therapeutics	Phase 3	NCT02255552	Studies targeting patients 7-16 years.	
Eteplirsen	PMO targeting exon 51	Sarepta Therapeutics	Phase 2	NCT02420379	Studies targeting younger patients, 4 to 6 years.	
Eteplirsen	PMO targeting exon 51	Sarepta Therapeutics	Phase 2	NCT02286947	Studies targeting patients in advanced stage, 7-21 years.	
SRP-4045	PMO targeting exon 45	Sarepta Therapeutics	Phase 1/2	NCT02530905		
SRP-4053	PMO targeting exon 53	Sarepta Therapeutics	Phase 1/2	NCT02310906		
NS-065/NCNP-01	PMO targeting exon 53	National Center of Neurology and Psychiatry (Japan)	Phase 1	NCT02081625		
Read-through						
Ataluren	Non-sense suppression	PTC Therapeutics	Phase 3 completed		Statistically non-significant, but 15 metre benefit in overall study population. Conditional drug approval in Europe in 2014.	www.ptcbio.com/
Ataluren	Non-sense suppression	PTC Therapeutics	Phase 3	NCT01557400, NCT02090959, NCT01247207	Extension studies on patients who previously have received Ataluren	
NPC-14	Arbekacin Sulfate	Kobe University (Japan)	Phase 2	NCT01918384		
Vector-mediated gene therapy						
Biostrongin	rAAV2.5-CMV-minidystrophin	Nationwide Children's Hospital (USA)	Phase 1 completed	NCT00428935	Safe, expression of dystrophin was limited and physical change was unremarkable.	[58]
	rAAVrh74.MCK.micro-Dystrophin	Nationwide Children's Hospital (USA)	Phase 1	NCT02376816		
Cell transplantation						
	HLA-identical allogeneic mesoangioblasts	Fondazione Centro s. Raffaele Del Monte Tabor (Italy)	Phase 1/2a completed	Eudract 2011-000176-33	Safe in four out of five patients, one had thalamic stroke of unknown relation. Inconclusive effect on muscle function.	[75]
	Autologous bone marrow mononuclear cell	Neurogen Brain and Spine Institute (India)	Phase 1	NCT02241434		
	Umbilical cord based allogenic mesenchymal stem cell	University of Gaziantep (Turkey)	Phase 1	NCT02484560		
	Umbilical cord mesenchymal stem cell	Acibadem University (Turkey)	Phase 1/2	NCT02285673		
	Autologous bone marrow derived mononuclear stem cell	Chaitanya Hospital, Pune (India)	Phase 1/2	NCT01834040		
	Normal myoblasts	Centre Hospitalier Universitaire de Québec, (Canada)	Phase 1/2	NCT02196467		

expected to restore the reading frame and be therapeutically applicable to 63% of DMD patients with deletion mutations, whereas single skipping of exon 51 could be applied to 16% of patients [25]. Furthermore, patients with exon 45-55 deletions were reported to have very mild myopathy symptoms [26]. In a mouse model of DMD harboring an exon 52 deletion, it was demonstrated that systemic antisense delivery could skip exons 45-55 to significantly improve muscle strength and histopathology [27]. Despite the many challenges to this approach, including cost, the technical challenges of skipping efficiency, and potential long-term toxicity, multi-exon skipping is an attractive approach that could extend the applicability of this therapy to a larger number of patients regardless of their mutation type.

The challenges that must be addressed by exon skipping strategies include the inability of ASOs to cross the plasma membrane without support, instability, a lack of cell specificity, and unwanted off-target effects. The chemistries that have been investigated other than 2'OMePS and PMOs include locked nucleic acid (LNA), peptide nucleic acid, tricycloDNA (tcDNA), cell-penetrating peptide-conjugated PMOs (PPMOs), PMO internalization peptide-conjugated PMOs (Pip-PMOs), and vivo-morpholinos (vPMOs), which are octa-guanidinium groups on dendrimeric scaffolds linked to morpholino oligos [10, 28, 29] (**Table 1**). LNA-modified ASOs exhibit better mismatch discrimination and high resistance to nucleases, tcDNA has an enhanced target-binding affinity and improved nuclease resistance [10, 30]; PPMOs, Pip-PMOs, and vPMOs contain cell-penetrating moieties [28]. Viral vectors and synthetic vectors (liposomes, cationic peptides and polymers, and protein complexes), as well as covalent attachments such as antibodies, peptides, lipids, carbohydrates, growth factors and vitamins have been tested to increase target-specificity [10]. Recently, we reported the accelerated cell uptake of amphiphilic PPMOs mediated by class A scavenger receptor subtypes (SCARAs) and that this mechanism is dependent on their self-assembly into nanoparticles [31]. Optimizing the self-assembly of PPMOs into nanoparticles may facilitate a next-generation technology for enhanced PPMO delivery.

Read-through therapy

Patients with nonsense mutations, which comprise 10% of all patients with DMD [9], can ben-

efit from read-through therapy. Nonsense mutations generate stop codons, thereby leading to premature translational termination and truncated proteins, and mRNA is destabilized by nonsense-mediated mRNA decay (NMD) [32]. The inactivation of any factor related to NMD pathway may stabilize transcripts generated from nonsense-containing coding regions. These observations indicate that nonsense-containing mRNA may produce a significant amount of functional protein by altering the decay rate or the extent of premature termination [32].

It is known that aminoglycoside antibiotics can cause phenotypic suppression in bacteria, which is because of disruption of the reading of ribonucleotides in polypeptide synthesis [33], thereby allowing the insertion of alternative amino acids at the site of the mutated codon [34]. Further studies have extended this process to mammalian cells [35], and the read-through of premature nonsense codons by aminoglycosides has been applied to nonsense mutation-mediated disease in cystic fibrosis and DMD, first in cultured cells and mouse models and then in patients [32]. *In vitro and in vivo* assays using gentamicin-treated *mdx* mice, an animal model of DMD harboring a nonsense mutation, demonstrated the restoration of membrane dystrophin expression and functional protection against contraction induced muscular injury [36]. Two studies of gentamicin treatment were conducted in a small number of patients with DMD and BMD, which demonstrated increased dystrophin production in one patient but no success in producing detectable full-length dystrophin or improved muscle strength in the other patients; thus, it was suggested that further studies with greater dosages and treatment lengths were warranted [37, 38]. Patient serum creatine kinase levels decreased after treatment in both studies, which was replicated in a later clinical trial with administration of gentamicin over a longer period [39]. In the latter study, patients treated for 6 months had significantly increased dystrophin levels in their post-treatment muscle compared with the pre-treatment muscle [39].

The results obtained supported the potency of gentamicin-mediated read-through, but the potential for renal and otic toxicities, as well as the lack of clear clinical efficacy led researchers to investigate novel compounds. High-throughput screening was performed to identify

compounds that promote nonsense suppression, where minimally toxic compounds were sought [32]. These analyses identified ataluren (formerly known as PTC124) (3-[5-(2-fluorophenyl)-[1,2,4]oxa-diazol-3-yl]-benzoic acid; C15-H9FN2O3), a compound that shares no structural similarity with aminoglycosides and is active at a lower concentration [32]. Ataluren selectively induces ribosomal read-through of premature but not normal termination codons and promotes dystrophin production in human and mouse muscle cells, as well as protecting the muscle from contraction-induced injury in *mdx* mice [32]. A phase 2a, open-label, sequential clinical trial was conducted in patients and a quantitative analysis assessing the dystrophin/spectrin ratio demonstrated increased dystrophin expression in 23 of 38 patients, while a qualitative analysis based on immunofluorescence detected positive changes in 13 of 38 patients [40]. A phase 2b, multicenter, randomized, double-blinded trial detected a nonsignificant but favorable result, where the difference in 6MWT was 31.3 m in favor of the treated group [41]. PTC therapeutics, the pharmaceutical company that developed ataluren, has recently announced the results of a phase 3, double-blind, placebo-controlled study, which detected a nonsignificant effect, although a 15-m improvement was observed in 6MWT in the overall study population, in addition to no loss of ambulation in the treated group compared with loss in 4 of 52 patients in the placebo group (<http://ir.ptcbio.com/releasedetail.cfm?ReleaseID=936905>).

Some researchers have expressed doubts over ataluren's mechanism of action and the European Medicines Agency (EMA), the drug regulator in the EU, previously rejected the drug for approval [42]. However, the urgent clinical need led the EMA to reconsider its approval, and ataluren (Translarna) has now received authorization for marketing in Europe.

Compounds other than ataluren have also been investigated, where RTC13 and RTC14, compounds with read-through function identified by high-throughput screening assay, have potential, but they have not been tested in patients [43].

Vector-mediated gene therapy

Originally, the identification of patients with very mild BMD with large deletions in the cen-

tral part of the *DMD* gene [11, 44] led to the suggestion that dystrophin containing only some domains could still be functional [45]. Research has shown that the central rod domain and C-terminal domain can be truncated with minimal impact on function but not the N-terminal domain or cysteine-rich domain deletions [46-48].

A general scheme for vector-mediated gene therapy in DMD is to deliver functional copies of the *DMD* gene to restore the lost protein via viral or nonviral vectors. Several viral vectors have been studied, including lenti- and adeno-associated viruses (AAVs) [45], and AAV vectors are currently the most widely used vector in vector-mediated gene therapy. The reason for the wide usage of AAV vectors refer to their low immunogenicity and defective replication, which facilitate organ-specific functional interventions, with long-term transgene expression over many years in nondividing cells [49, 50]. Additionally, the wild-type AAV is not associated with any known pathogenicity [49]. Microdystrophins are functional, smaller cDNA clones of dystrophin with four or fewer repeats [51], and the small size of micro-dystrophin allows it to be packaged into AAV vectors with a relatively small packaging capacity of about 5 kb [49]. When AAV vectors packaged with micro-dystrophin was injected locally to the muscle of *mdx* mice, dystrophin and DGC was restored, as well as ameliorated muscle histopathology [50, 51]. Furthermore, micro-dystrophin could restore muscle function [52, 53]. Systemic AAV gene transfer in larger animals, that is, DMD dogs, has also demonstrated the safe and efficient transduction of the *DMD* gene [54]. On the other hand, an attempt to transfer the full-length *DMD*-coding sequence into muscle cells by dual high-capacity adenovirus/AAV vectors has been reported [55]. More recent reports have demonstrated the delivery of the full-length *DMD* gene via multiple AAV vectors in *mdx* mice by splitting the full coding sequences into three fragments and packaging them into separate independent vectors, which can be reconstituted into a larger clone *in vivo* [49, 56, 57].

In human DMD trials, a local injection study of full-length dystrophin delivery demonstrated the expression of dystrophin, but the low reconstitution rate and ambiguous functional benefit are problems that still need to be solved. A

phase 1, randomized, double-blind, placebo-controlled clinical study of the local injection of AAV-mini-dystrophin demonstrated its safety and it was well tolerated, while AAV genomes were detected in all of the patients. However, the expression of dystrophin was limited, and the changes according to physical examinations were unremarkable [58].

It has been reported that clinical study of gene replacement via a self-complementary AAV9 vector in spinal muscular atrophy type 1 patients, which is a neuromuscular disorder that manifests severe muscle weakness from early infancy, yielded significant improvements in survival (NCT02122952). Further, an AAV product have already been approved for marketing in Europe, which is Glybera, the AAV vector engineered to express lipoprotein lipase to treat lipoprotein lipase deficiency [59]. Taken together, AAV vector-mediated gene delivery is a promising therapeutic approach.

Two main concerns need to be resolved regarding the immune response to AAV vector-mediated gene therapy. One is the potential pre-existence of AAV-neutralizing antibodies because a significant proportion of the human population is known to have pre-existing neutralizing antibodies for AAV [60]. The second is the potential risk of inducing immunoreactivity to the newly translated dystrophin obtained from the therapeutic transgene, as reported by Mendell *et al.* [61]. Both pre-existing neutralizing antibodies for AAV and induced immunoreactivity to newly translated protein can lead to loss of transgene expression [60-62]. The usage of immunosuppressive therapy and other preventive measures may be required to induce persistent antigen-specific tolerance [63].

Nonviral vectors such as plasmids, human artificial chromosomes, and transposons have the advantages that they can avoid any risk of an immune response because of viral proteins and insertional mutagenesis, and most have a high cloning capacity [45]. A sequential study of locally injected plasmid-based gene therapy in nine patients with DMD/BMD detected no side effects, and dystrophin expression was indicated in six patients [62].

Cell transplantation

The transplantation of cells that produce functional dystrophin into patients with DMD can

restore the lost protein, as well as lead to the formation of new fibers. The cells used for transplantation can either be genetically unmodified cells from normal donors or be autologous genetically corrected cells [64], and the cell source can be variable.

Satellite cells (SCs), which were identified in the 1960s, are cells located underneath the basal lamina of muscle fibers [65], and they are considered to be the stem cells for skeletal muscle. SCs are present in mammalian muscle as quiescent cells and when activated by muscle injury they transform into myoblasts and generate large numbers of new muscle fibers [66]. SCs and myoblasts are considered to be good candidates for cell therapy in DMD because of their ability to generate and regenerate muscle fibers. Normal myoblasts injection into *mdx* mice converted pre-existing or regenerated muscle fibers as dystrophin positive [67]. In patients with DMD, the intramuscular injection of normal human SCs or myoblasts into a small number of patients led to the expression of donor-derived dystrophin to some extent, but no clear data on the amelioration of muscle function were reported [68-70].

Cell sources other than SCs and myoblasts include muscle-derived stem cells, CD133+ stem cells, PW1+ interstitial cells, mesoangioblasts, mesenchymal stem cells, hematopoietic stem cells, embryonic stem cells, and induced pluripotent stem (iPS) cells. Among these, the transplantation of CD133+ stem cells and mesoangioblasts are the most advanced forms of cell therapy currently in clinical trials conducted in patients. CD133, which was originally described as a polypeptide expressed on a population of circulating human hematopoietic/endothelial progenitors, was identified as comprising a subpopulation of human muscle-derived stem cells that could differentiate into muscle, hematopoietic, and endothelial cell types when exposed to certain cytokines [71]. Injecting human circulating CD133+ stem cells into the skeletal muscle of *scid/mdx* mice was shown to significantly ameliorate the muscle structure and function [71]. A phase 1, double-blind clinical trial based on the autologous transplantation of muscle-derived CD133+ stem cells into patients with DMD was found to be safe [72].

Mesoangioblasts are vessel-associated stem cells [73]. In skeletal muscle, they can be iso-

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Table 3. Descriptions of registered clinical trials for studies aiming to compensate for the lack of dystrophin. (Current status, December 2015)

Drug/compound	Description	Company/Institute	Status	Clinical trial no.	Remarks	Ref.
Anti-inflammatory/fibrotic/oxidant						
HT-100	Halofuginone	Akashi Therapeutics	Phase 1/2	NCT01847573, NCT01978366, NCT02525302		
CATENA/RAXONE	Idebenone	Santhera Pharmaceuticals	Phase 3 completed	NCT01027884	Shown reduction in loss of respiratory function.	[83]
CAT-1004	EPA, DHA	Coordinación de Investigación en Salud (Mexico)	Phase 2	NCT01826422		
	Salicylate and DHA conjugate	Catabasis Pharmaceuticals	Phase 1/2	NCT02439216		
TAS-205	Epigallocatechin-Gallate	Charite University, Berlin, Germany	Phase 2/3	NCT01183767		
	Prostaglandin D synthase inhibitor	Taiho Pharmaceutical Co., Ltd.	Phase 1 completed	NCT02246478		
Myostatin pathway inhibitor						
	rAAV1.CMV.huFollistatin344	Nationwide Children's Hospital (USA)	Phase 1/2	NCT02354781		
Givinostat	Histone deacetylases inhibitor	Italfarmaco	Phase 1/2	NCT01761292		
PF-06252616	Anti-myostatin monoclonal antibody	Pfizer	Phase 2	NCT02310763		
nNOS pathway enhancement						
LY450190/Cialis	Tadalafil	Eli Lilly and Company	Phase 3	NCT01865084		
	Tadalafil and sildenafil	Cedars-Sinai Medical Center (USA)	Phase 1 completed	NCT01580501	Improved functional ischemia and exercise-induced muscle blood flow.	[100]
	L-citrulline and Metformin	University Hospital, Basel (Switzerland)	Phase 3	NCT01995032		
	L-Arginine and Metformin	University Hospital, Basel (Switzerland)	Phase 1	NCT02516085		
Utrophin up-regulation						
SMT C1100	5-(ethylsulfonyl)-2-(naphthalene-2yl)benzo[d]oxazole	Summit Therapeutics	Phase 1b completed	NCT02056808		

lated from the muscle vasculature, and they can differentiate into muscle fibers which migrate from the vessel into the surrounding muscle fibers where they eventually differentiate into local tissues [73]. The intra-arterial delivery of wild-type mesoangioblasts in DMD dogs led to the recovery of dystrophin expression, muscle morphology and muscle function [74]. Recently, a phase 1-2a, nonrandomized, open-label clinical trial of intra-arterial mesoangioblast transplantation in five patients with DMD was reported to be relatively safe, where one patient developed a thalamic stroke with an unknown relationship to the therapy but with inconclusive effects on muscle function [75].

The utilization of iPS cells, which are still at the preclinical investigation stage, is expected to be one of the most promising strategies for future applications. Indeed, the transplantation of human iPS-derived myogenic cells into *mdx* mice had favorable outcomes in terms of dystrophin expression and muscle function [76].

Therapies that compensate for the lack of dystrophin

Reports of animal models and patients with both out-of-frame mutations in the *DMD* gene and the complete lack of dystrophin in muscle specimens with minimal muscle weakness have attracted researchers to using genetic and epigenetic disease-modifying factors. The potential of environmental circumstance modulation in DMD may lead to the dramatic amelioration of this phenotype.

Anti-inflammatory and anti-fibrotic agents/antioxidants

Muscles from patients with DMD exhibit the infiltration of inflammatory cells, predominantly by macrophages and lymphocytes [77]. M1 and M2 macrophages, which are both observed in the muscles of patients with DMD, have been reported to play individual roles, where M1 macrophages occur acutely to phagocytize cell debris and the M2 macrophages then regenerate myofibers [77, 78]. In addition, several other immune cells and cytokines regulated by nuclear factor-kappa B (NF- κ B), a major inflammatory and transcription factor, are known to be involved in the pathophysiology of DMD. A study that depleted macrophages from *mdx* mice reported reductions in the muscle pathology

[79]. Together with evidence of the positive effects of glucocorticoids in patients with DMD, the rationale for pursuing the anti-inflammatory approach in DMD therapy has come into clearer focus.

Endomysial fibrosis in the muscles of patients with DMD is correlated with clinical severity and because the transforming growth factor- β (TGF- β) pathway plays an important role in fibrotic tissue formation, this has now become the major target of antifibrotic approaches in DMD [80, 81]. Increases in reactive oxygen species have also been found in DMD, which are considered to contribute to membrane permeability, protein degradation, and inflammatory pathway activation [82], and thus they are potential therapeutic targets.

A recent phase 3 clinical trial using idebenone, an antioxidant that inhibits lipid peroxidation and is capable of stimulating mitochondrial electron reflux as well as cellular energy production, demonstrated the reduced loss of respiratory function in patients with DMD [83]. Other drugs such as halofuginone, a TGF- β -mediated pathway inhibitor [84], and epigallocatechin-gallate, a polyphenol that is a potential antioxidant, are also in clinical trials (**Table 3**). Additionally, the clinical trials on CAT-100, a conjugate of salicylate and docosahexaenoic acid acting as an NF- κ B inhibitor (<http://www.catabasis.com/>), and a prostaglandin D synthase inhibitor [85] are underway (**Table 3**).

Myostatin pathway inhibitor

Myostatin is a member of the TGF- β superfamily that is essential for the appropriate regulation of skeletal muscle mass, and it was first reported in 1997 [86]. When the *Myostatin* gene is disrupted, the skeletal muscle of myostatin-null mice becomes significantly larger than that in the wild type, which is a consequence of the increased number and size of the muscle fibers [86]. In addition, it has been indicated that inhibiting the function of myostatin may be useful in the treatment of musculodegenerative diseases including muscular dystrophies. Animal studies with *mdx* mice that genetically lacked or that had postnatally blocked myostatin exhibited improved muscle phenotypes [87, 88]. Activin receptor type IIB, a receptor of TGF- β superfamily members including myostatin and follistatin, an activin-binding

protein that inhibits myostatin from binding to the receptors, have also been studied in *mdx* mice with favorable outcomes [89, 90]. Clinical trials of *Follistatin* gene transfer via AAV vector, follistatin expression induction by deacetylase inhibitors [91], and anti-myostatin monoclonal antibody administration are currently under way (Table 3).

Neuronal nitric oxide synthase (nNOS) pathway enhancement

There are three major isoforms of nitric oxide (NO), which are neuronal NOS (nNOS), endothelial NOS, and inducible NOS. Among the three isoforms, nNOS is the most prominent type expressed in skeletal muscle, where it is a component of DGC and it is the predominant source of NO. Alternative splicing yields two functionally distinct nNOS forms; that is, nNOS μ localizes to the sarcolemma and nNOS β localizes in the Golgi [80, 92]. The NO produced by nNOS μ attenuates vasoconstriction to deliver adequate blood and oxygen to the muscle, whereas the NO generated via nNOS β maintains the ability of contracting muscle to generate force during and after exercise [80].

NO stimulates soluble guanylate cyclase to produce cyclic guanosine monophosphate (cGMP), which acts to relax smooth muscle in the vasculature. Studies have shown that in dystrophin-deficient muscle nNOS is aberrantly translocated from the sarcolemma to the cytosol, while the overall nNOS level is decreased compared with that in normal muscle [93, 94]. These observations have attracted interest in nNOS because of its role in pathogenesis but also as a potential target for therapeutic strategies.

It has been proposed that the ischemic process plays a role in the pathogenesis of muscle fiber necrosis in DMD [95]. It was also reported that the vasoconstrictor response to reflex sympathetic activation was defective during exercise in nNOS null mice, *mdx* mice, and patients with DMD but not in other nNOS-intact muscle diseases. Therefore, nNOS deficiency may lead to functional muscle ischemia as well as contribute to the pathogenesis of DMD [96, 97].

The administration of phosphodiesterase-5 inhibitor (PDE5I), which can increase the intracellular cGMP level in vascular smooth muscle cells and cause vasodilation, was shown to

have a beneficial effect in *mdx* mice [98]. Another study demonstrated that genetically-overexpressed cGMP production in the hearts of *mdx* mice reduced cardiomyopathic changes [99]. A phase 1, open-label, crossover trial with PDE5I (tadalafil or sildenafil) in patients with DMD indicated the alleviation of functional ischemia and normalized exercise-induced increases in muscle blood flow [100]. In addition, a phase 2, double-blind, randomized, placebo-controlled study of sildenafil found no improvement in cardiomyopathy in DMD [101].

Utrophin upregulation

Utrophin, a dystrophin homologue, was discovered in a study of proteins that share sequence similarity with dystrophin C-terminal domain [102]. In healthy skeletal muscle, utrophin was observed at neuromuscular junctions, but utrophin was upregulated at the sarcolemma in muscles from patients with DMD and *mdx* mice [103]. In a therapeutic context, the genetic upregulation of utrophin and the subsequent development of an orally bioavailable small molecule (SMT C1100) to upregulate utrophin led to improved muscle pathology and function in *mdx* mice [104-106]. SMT C1100 was developed in a high-throughput screening assay to identify compounds with the ability to increase the transcription of endogenous utrophin using a human muscle-specific utrophin A promoter cell-based assay [106]. A phase 1a clinical study of SMT C1100 in healthy volunteers demonstrated its safety [107], and another study using a second-generation compound from the SMT C1100 family with improved physicochemical properties and more robust metabolism profiles obtained a favorable outcome in *mdx* mice [108].

The main concern related to utrophin-targeted therapy is that utrophin does not anchor nNOS to the sarcolemma, and thus unmet metabolic needs related to blood flow defects in DMD may remain [109].

Future perspectives

The innovative therapeutic approaches illustrated in this review are supported by a rationale and accumulated preclinical data. One of the most promising therapies is arguably ASO-based exon skipping. However, the main problem of the current ASO drug-based approach is

its limited efficacy owing to poor cellular uptake. The hydrophobic plasma membrane constitutes an almost insurmountable barrier around muscle fibers, resulting in poor delivery of ASO and preventing optimization of ASO-mediated therapy for DMD or other muscular dystrophies. In regard to this issue, we have recently reported that PMO and 2'OMePS entries into muscle fibres are dependent on the myogenic stage rather than on an intrinsic defect in the membrane of muscle fibres lacking dystrophin: the "leaky-membrane" hypothesis [110]. Furthermore, we have demonstrated scavenger receptor-mediated uptake for a range of therapeutic ASO chemistries depending on their self-assembly into nanoparticles. The success in understanding the uptake mechanism of ASOs provides a platform for designing an entirely new ASO with efficient cellular uptake.

In parallel to the work on ASO-based exon skipping studies, intensive pre-clinical research for DMD therapy is underway. Recent data showed significant amount of evidence on clustered regularly interspaced short palindromic repeats (CRISPR)-based genome editing for *DMD*. CRISPR system is a gene editing technology evolved from the adaptive immune system found in microbes to defend themselves against invading viruses by targeting their DNA sequences [111-113]. *Mdx* mice were treated with CRISPR/Cas9 system delivered by AAV vector, and have shown restoration of dystrophin in muscle fibers and improvement in muscle function [114-116]. In addition, this CRISPR-based gene editing in *mdx* mice restored dystrophin in cardiomyocytes. Other studies using animal models have indicated the possibility of long-term muscle regeneration through the granulocyte colony-stimulating factor (G-CSF) receptor axis [117], potential effect of gene editing by CRISPR/Cas9 [118], and marked amelioration of the phenotype by over-expressing the *Jagged1* gene [119]. These findings may also shed light on novel avenues of DMD therapy.

Irrespective of the great efforts made to develop novel therapies by basic scientists and physicians, many of these approaches have failed to exhibit the same efficacy in patients as shown in animal models. Several factors may explain why it is so difficult to replicate preclinical research data in patients. One is the small

number of patients of DMD, therefore, this issue hinders the design of large cohort studies with sufficient power to detect the efficacy of therapies. The second main problem is the heterogeneity in the severity of DMD, which may also be a factor that prevents comparisons of the functional outcomes between the treated and placebo groups. The third issue is the lack of optimal outcome measures to evaluate the efficacy of new drugs for DMD. For example, compelling argument has been provided for the clinical use of 6MWT in the evaluation of patients with DMD. In addition, 6MWT cannot be applied to patients who are non-ambulatory. Surrogate biomarker such as dystrophin also has limitation, since the assessment of dystrophin levels in a small piece of muscle represents only a crude estimate of clinical efficacy. It might be fundamental to understand the natural history of DMD and its covariates including gene mutations, which can influence the sensitivity of clinical trials, will help in the design of future clinical trials [120]. Together, the development of a therapeutic approach and outcome measures, including biomarkers, with more versatility is urgently required.

Conclusion

Over recent years, there has been great progress in DMD therapy development.

With the advances in the innovative therapeutic approaches, the development of clinical trial networks and optimization of outcome measures for evaluating clinical efficacy have been conducted. As exemplified in TREAT-NMD (<http://www.treat-nmd.eu/>) or MDCTN (<http://www.mdctn.jp/>) in Japan, the clinical trial networks have made more accessible to resources and expertise, thereby making the researchers and pharmaceutical companies much easier to conduct clinical trials. These platforms have accelerate the path for pre-clinical discoveries to be utilized in clinical settings and are not only applicable to DMD but also to other neurological and muscular disorders that have no cure.

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Disclosure of conflict of interest

None.

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