The Pathogenesis and Therapy of Muscular Dystrophies

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Abstract

Current molecular genomic approaches to human genetic disorders have led to an explosion in the identification of the genes and their encoded proteins responsible for these disorders. The identification of the gene altered by mutations in Duchenne and Becker muscular dystrophy was one of the earliest examples of this paradigm. The nearly 30 years of research partly outlined here exemplifies the road that similar current gene discovery protocols will be expected to travel, albeit much more rapidly owing to improved diagnosis of genetic disorders and an understanding of the spectrum of mutations thought to cause them. The identification of the protein dystrophin has led to a new understanding of the muscle cell membrane and the proteins involved in membrane stability, as well as new candidate genes for additional forms of muscular dystrophy. Animal models identified with naturally occurring mutations and developed by genetic manipulation have furthered the understanding of disease progression and underlying pathology. The biochemistry and molecular analysis of patient samples have led to the different dystrophin-dependent and -independent therapies that are currently close to or in human clinical trials. The lessons learned from decades of research on dystrophin have benefited the field of human genetics.

INTRODUCTION AND *DMD* GENE IDENTIFICATION

The gene and protein involved in Duchenne muscular dystrophy (DMD) were identified more than 25 years ago (104, 125). These discoveries facilitated important research into the disease mechanisms of DMD and other muscle diseases and initiated therapy development. In this review, we outline the process of gene and protein identification, discuss therapies currently being developed for DMD, and describe the tools required for translational research. We regret being unable to refer to some of the work that has been reported so far owing to space constraints.

DMD is a severe X-linked recessive disorder with an incidence of 1:5,000 (163), making it one of the most common recessive disorders in the human population. The disease presents in boys at an early age, with diagnosis at approximately four years of age. Patients show progressive muscle wasting and weakness, leading to wheelchair dependency usually by the age of 10, assisted ventilation before the age of 20, and premature death in the second to fourth decade. One of the hallmarks of this progressive muscle wasting disease is the histological appearance of the muscle. Normal muscle, when stained with hematoxylin and eosin, is highly organized with regular-sized fibers. By contrast, patients with DMD show a disorganized muscle with hypertrophy of myofibers, inflammation, and extensive deposits of connective tissue and fat (70).

The question in the early 1980s was how one could identify the gene responsible for this disorder without a known biochemical defect. The disorder was clearly genetic, but unlike other diseases, such as the hemoglobinopathies and Lesch-Nyhan syndrome, there were no clues to the identity of the defective protein. The gene was known to reside on the X chromosome because generally only boys were affected, although occasionally their mothers presented with milder symptoms as symptomatic carriers. Intriguingly, at this time, a small number of girls with balanced X/autosome translocations were reported, which suggested that the gene might be at Xp21 (33, 227, 245). In these cases, the translocated X chromosome remains active, and the normal X chromosome is inactive. The hypothesis was that the translocation at Xp21 disrupted the gene mutated in DMD.

Botstein et al. (30) proposed the key to making progress in mapping unknown genes and the genome itself: Using linked sequence variants that are themselves insignificant but are detectable as restriction fragment length polymorphisms (RFLPs), one could track disease genes in families to their (sub)chromosomal locations. Through this approach, linkage to RFLP markers showed that the *dystrophin* (*DMD*) gene indeed resides on the short arm of the X chromosome (66). Harper and colleagues (121) subsequently mapped the less common and milder form of the disease, Becker muscular dystrophy, to the same region of the X chromosome. These RFLP markers were used to perform prenatal diagnosis for women who were at risk of carrying *DMD* mutations (14). This was an important step because until then, no prenatal test had been available. Carrier detection was based on serum creatine kinase levels (a marker for muscle damage), which are increased in ~50% of carriers, and this method was therefore not very reliable.

The defective gene was identified using two independent approaches. The first took advantage of the X/autosome translocation, which disrupted the ribosomal RNA genes on chromosome 21. Worton et al. (239) proposed using ribosomal RNA genes to isolate the breakpoint clone of the translocation between chromosome 21 and the X chromosome, which should also contain part of the *DMD* gene from the X chromosome. A second approach took advantage of patient BB, who was first reported and characterized cytogenetically by Francke et al. (82). This patient was a boy exhibiting four X-linked disorders: DMD, chronic granulomatous disease, retinitis pigmentosa, and the rare McLeod red cell phenotype. The reasoning at the time was that his disorders represented a contiguous gene deletion syndrome. Indeed, one of the DNA markers for the Xp21 region, called 754 and isolated by van Ommen and coworkers, was absent from BB's DNA, proving the existence of a deletion.

Using a competitive hybridization strategy between DNA isolated from BB and an individual with four X chromosomes, Kunkel et al. (133) identified small DNA fragments that were also missing from BB's DNA, documenting the specific cloning of DNA segments from the DNA isolated from the boy. Combined with 754, the eight cloned segments contained DNA from the X chromosome that presumably mapped near or within the four genes, causing BB's complex phenotype.

It was known that approximately 5% of patients who have an X-linked disorder have a small deletion as the cause of it. The sequences known to be in the BB deletion were therefore investigated for their absence in DMD patients on the basis that they might reside in the *DMD* gene. One clone, pERT87 (designated as the *DXS164* locus), was able to detect deletions in DMD patients (171). In parallel, a ribosomal junction fragment called XJ, cloned from a translocation carrier female, also detected deletions (186). These clones were potentially detecting the primary mutation in these DMD patients. Given the severity and relatively high prevalence of DMD, physicians, parents, and patients were anxious for the ability to perform accurate prenatal diagnosis and carrier detection. The pERT87 clone was made available to many investigators around the world so that a rapid assessment could be made of how frequently these deletions occurred in patients. A then-unique collaboration involving 75 authors analyzed more than 1,300 DMD patients for deletions, and approximately 8% were found to be deleted for the pERT87 clone (132).

Mapping the deletion breakpoints in the chromosome walk around pERT87/*DXS164* identified many patients who had parts of the locus present and parts missing, in both directions. Several of the *DXS164* subclones detected RFLPs; surprisingly, using these to track the *DMD* gene in families recombination mapped the disease in each separate family either distally or proximally to the same markers. This strongly suggested that the *DMD* locus was large and that the mutations could lie on both sides outside *DXS164*. The fact that the *XJ* locus was completely independent of *DXS164* indicated that these regions either were located in more than one causative gene or were part of one gene spaced over a very large distance (39).

One surprising result emerged from these studies: Although it was well known, based on the classical rule of Haldane, that one in every three cases of DMD was due to a new mutation, several noncarrier mothers of new-mutation DMD patients turned out to carry a second pregnancy with an identical mutation (15). This indicated that the mutation had not arisen spontaneously in one of the mother's oocytes but rather had appeared earlier in germline maturation and had then been propagated to a fraction of germ cells. This so-called germline mosaicism was found to affect 14% of oocytes of mothers of new-mutation patients. This phenomenon, which complicates carrier detection and prenatal diagnosis, was subsequently found to be generally associated with new mutations.

Monaco et al. (172) used conservation among species to further track down the gene in the large segment of genomic DNA sequence, on the basis that coding sequences for a protein might have sufficient nucleotide homology among the species to be picked up by Southern blot hybridization. A cloned DNA segment from the *DXS164* locus showed species conservation, and when used as a hybridization probe against a muscle cDNA library, it was able to pick out a cDNA that hybridized to eight HindIII restriction fragments in human DNA. All of the hybridizing HindIII fragments were completely missing from DNA isolated from patient BB, and they were spaced over the entire 210-kb region of *DXS164* (172).

On northern blots, the transcript that was detected by the muscle cDNA was 14 kb in size. This, combined with the spacing of exons in the *DXS164* locus, provided further evidence that it was a very large locus. In parallel, chromosome walking and sequence conservation studies of the *XJ* region also identified a second set of cDNAs that did not overlap with *DXS164*, again implying a very large locus (39).

A new technique for physical mapping, pulsed field gel electrophoresis, confirmed that the *DMD* locus was indeed large (42, 117, 226). Mapping of several deletions in patients showed that the *DMD* gene covers more than 2 million base pairs (225). More recent studies combining DNA and RNA mapping and sequencing have shown that it consists of 79 exons covering approximately 1% of the X chromosome (RefSeq NG_012232.1). As such, the *DMD* gene is the largest mammalian locus encoding a single set of protein isoforms. The large size of the locus makes it a target for deletion mutations, and the majority of mutations at the locus are indeed deletions (65%) or duplications (11%) (26), with the remainder being small mutations affecting the coding sequence and splice sites. However, these deletions occur predominantly in two hot spots of the gene (81, 124).

Interestingly, both the milder Becker muscular dystrophy and more severe DMD are caused primarily by mutations in the *DMD* gene, the difference being the predicted effect the mutations have on the translational reading frame of the dystrophin transcript. Becker patients carry in-frame mutations (which can involve many exons) and produce an internally truncated protein that can have nearly normal function. By contrast, DMD patients carry out-of-frame mutations, or nonsense mutations that lead to premature truncation of protein translation and nonfunctional dystrophin (170).

During the screening of muscle cDNA libraries for the isolation of the full-length *DMD* gene, Davies and colleagues (147) identified a cDNA fragment that was highly homologous to the *DMD* gene but mapped to human chromosome 6. This gene turned out to be large as well and to consist of an intron-exon structure that is very similar to that of the gene encoding dystrophin, suggesting that the two genes are probably related by an ancient duplication event (182). The dystrophin-related protein encoded by this gene is expressed in most tissues, and it was postulated that it might act as a surrogate for dystrophin in muscle (119, 147).

DYSTROPHIN BIOCHEMISTRY AND FUNCTION

The identification of the dystrophin protein (so named because its lack results in muscular dystrophy) led to a whole new outlook on the muscle cell membrane and its interaction with the extracellular matrix. The cDNA open reading predicted a 427-kDa protein, which was detected with a series of antibodies directed against bacteria-expressed fusion proteins encoded by candidate cDNA clones (104). Upon western blotting, the 427-kDa protein was originally shown to copurify with membranes, and those membrane preparations were thought to be most enriched with the triad structures of muscle (105). Immunohistochemistry later showed that dystrophin seemed to be localized not to the triads but to the plasma membrane (9, 29, 246). More recent studies have documented the presence of dystrophin at the T-tubules of the triad structures (96, 122).

Having antibodies directed to dystrophin led to the purification of dystrophin from membrane preparations of skeletal muscle and to the identification of a novel group of proteins that copurified with dystrophin. The first protein found to interact with dystrophin was dystroglycan (74). Later, the same group showed that there are actually several other proteins that interact with dystrophin, and they proposed a model of this interaction termed the dystrophin-associated protein complex (DAPC) (47, 72, 109, 240, 241). Using a slightly different biochemical purification approach, another group was able to show that the dystrophin-associated proteins actually separate into two classes: the dystroglycan complex and the sarcoglycan complex (153, 240, 242). Over the years, the study of dystrophin and the DAPC has evolved, and more and more proteins have been shown to associate with dystrophin either directly or via dystroglycan (109, 242) (see **Figure 1**). Many of these proteins are encoded by genes that harbor mutations causing other forms of muscular dystrophy.

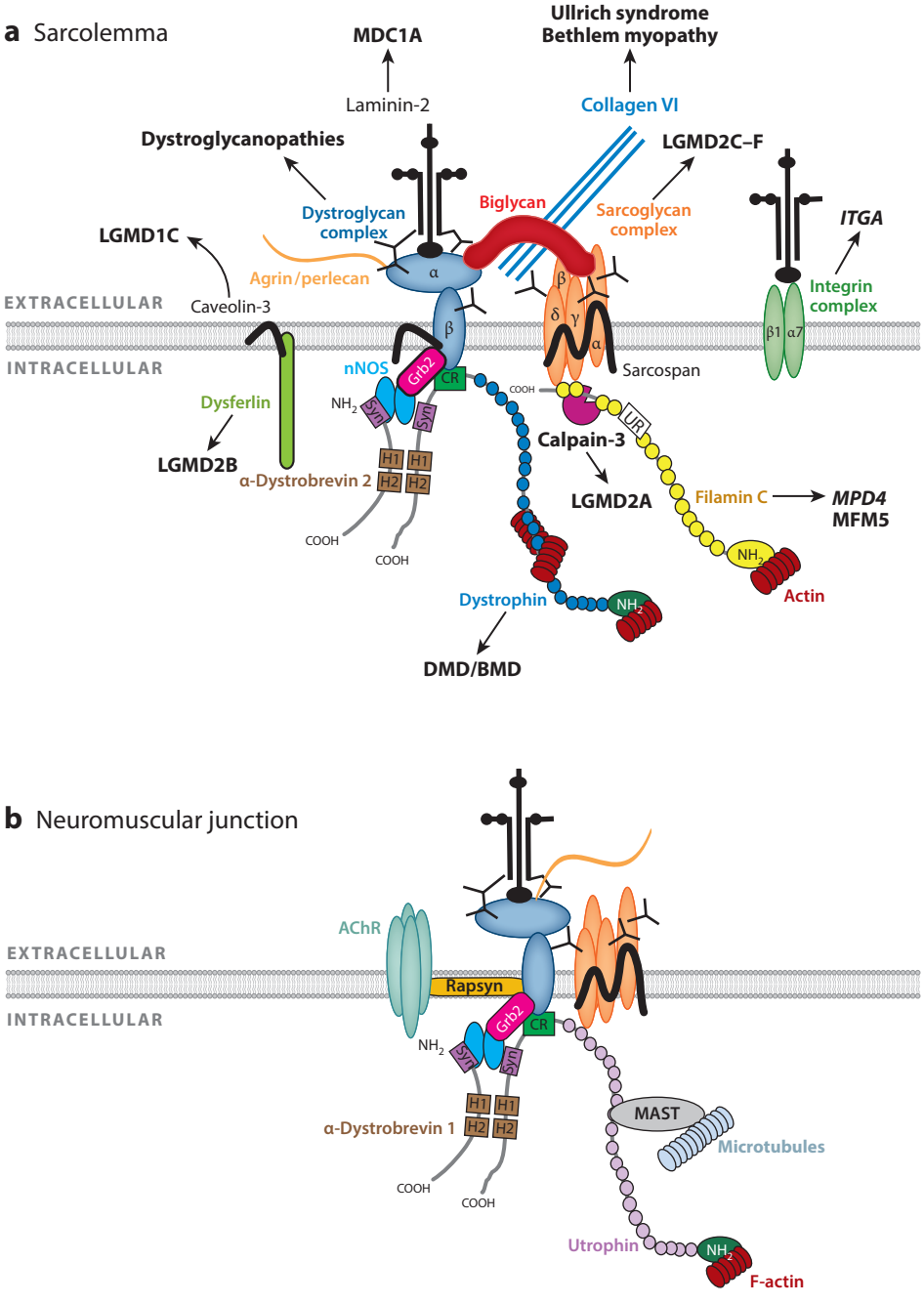
Dystrophin is localized to the inner surface of the muscle cell membrane and is enriched at the costameres and sites of cell-cell contact (212). It has four major domains: an N-terminal region with homologies to the actin-binding domains of α-actinin; a central rod domain composed of spectrin-like repeats broken by four hinge regions; a cysteine-rich domain that comprises a WW domain, two EF-hand-like domains, and a ZZ domain; and a C-terminal domain (127). The N terminus of dystrophin contains the predicted actin-binding domain that is responsible for anchoring dystrophin to the cytoskeleton, yet additional studies have shown that the actin binds along much of the N-terminal half of dystrophin (73, 193). The central rod domain of dystrophin contains spectrin-like repeats (126). The rod region has a second site for binding γ-actin, and this site differs considerably from the dystrophin homolog utrophin. The cysteine-rich region at the C terminus is connected to the DAPC at the sarcolemma through interaction with β-dystroglycan (107, 110). The extreme C-terminal region is α-helical in nature and mediates its interaction with the syntrophins. Therefore, dystrophin connects the sarcolemma to the actin cytoskeleton, playing an important role in muscle cell membrane stability. The connection is achieved through the DAPC, which consists of cytoplasmic [α1- and β1-syntrophin, α-dystrobrevin, and neuronal nitric oxide synthase (nNOS)], transmembrane (β-dystroglycan; α-, β-, γ-, and δ-sarcoglycan; and sarcospan) and extracellular proteins (α-dystroglycan and laminin-2), providing a strong link between the intracellular cytoskeleton and the extracellular matrix.

Dystroglycan is the main protein in the DAPC whose two subunits are encoded by a single gene and posttranslationally cleaved. The transmembrane subunit, β-dystroglycan, binds to dystrophin at the intracellular periphery of the sarcolemma (110). The cell surface subunit, α-dystroglycan, binds to β-dystroglycan and to the extracellular matrix proteins, such as laminin-2; together, these proteins connect the intracellular cytoskeleton through the sarcolemma with the basement membrane (109). α-Dystroglycan is highly glycosylated, and many of the enzymes involved in this glycosylation are themselves mutated in other forms of limb-girdle muscular dystrophy and congenital muscular dystrophy. The underglycosylated α-dystroglycan is thought to not interact strongly enough with the extracellular matrix, thus compromising the structural link between the actin cytoskeleton and the extracellular matrix and leading to myofiber degeneration. The elucidation of the DAPC function and its localization at costameres linking the extracellular matrix and the cytoskeleton led to the hypothesis that the DAPC is critical for the stabilization of the sarcolemma during muscle contraction (73).

Although the structural role of dystrophin is important, there is clear evidence that dystrophin and the DAPC are also involved in signal transduction, likely via the association of nNOS with the complex and possibly via the sarcoglycan complex. The spectrin-like repeats at dystrophin predict that dystrophin might work as a shock absorber, helping to resist repeated rounds of muscle contraction and relaxation (126). Dystrophin is also phosphorylated both in vivo (167) and in vitro, and the C-terminal region has many predicted phosphorylation sites. Dystrophin dephosphorylation occurs by calcineurin (protein phosphatase type 2B) (166). The phosphorylation of dystrophin's rod domain affecting the F-actin-binding domain alters its affinity for actin (200), whereas phosphorylation of the dystrophin C-terminal domain inhibits syntrophin binding (149), and phosphorylation of a specific serine residue within the dystrophin WW domain increases the association between dystrophin and β-dystroglycan (213). Dystrophin is also the target of a variety of kinases, including mitogen-activated protein kinase (204), calmodulin-dependent protein kinase II, $p34^{cdc2}$ kinase (167), and casein kinase (148). However, the in vivo consequences of these phosphorylation events still need to be elucidated.

The enzyme nNOS is important to increase blood flow in muscles. nNOS is associated with the DAPC through binding to dystrophin spectrin repeats 16 and 17 (134) and through PDZ domain interactions with syntrophin (35). The enzyme activity leads to the production of nitric oxide

(NO) (34), which can inhibit or activate heme-containing proteins (238); these proteins, in turn, regulate contractile function, glucose metabolism, and calcium mobilization (92). nNOS plays a role in DMD pathogenesis: When its activity is reduced, the vasodilation in muscle is inhibited, which causes ischemia (195). Patients with DMD also show abnormal blood vessel constriction presumably caused by a lack of nNOS at the sarcolemma. Although nNOS has an important role in muscle function, the muscular dystrophy pathology is not dependent on nNOS (63).



Skeletal muscle is a dynamic tissue that normally undergoes mechanical stress from each contraction. The bond between the cytoskeleton of the muscle cell and the extracellular matrix is important to maintain function during the cycles of contraction and stress. Even normal skeletal muscle is susceptible to mechanical damage during contraction, which can cause defects in the sarcolemma (157) and T-tubules (111). The repetitive contractions cause injury that can lead to muscle degeneration and infiltration of inflammatory cells (56). In a normal muscle, this injury can be repaired by the activation of muscle stem cells and satellite cells, maintaining the homeostasis. However, because the DAPC serves as a physical connection of the sarcolemmal cytoskeleton with the extracellular matrix, the loss of this structural linkage is expected to make the sarcolemma more susceptible to damage when exposed to mechanical stress. Contraction in dystrophic muscle cells produces membrane damage, which leads to an increase of the sarcolemma permeability to calcium and small molecules, resulting in cell dysfunction and death (59). The continued cell death causes an imbalance between muscle degeneration and regeneration of skeletal muscle, leading to inflammatory responses and bursts of cytokine expression, which contribute to the fibrosis observed as the disease progresses. This leads to the disease pathology and consequently results in a progressive decline of muscle function from the loss of muscle myofibers and their replacement by connective tissue and fat. Any treatment that can alter the rate of these regeneration and degeneration cycles could have a profound influence on disease progression.

ANIMAL MODELS

To explore treatment avenues, animal models are needed, and these should mimic the human disorder. An ideal animal model should have the same genetic basis and reiterate key hallmarks and progression of the human pathology, have a robust and reproducible phenotype over generations,

Figure 1

(*a*) Composition and structure of the dystrophin-associated protein complex (DAPC) at the sarcolemma. Dystrophin contains N-terminal (NTD), central rod, cysteine-rich (CRD), and C-terminal (CTD) domains. The central rod domain contains 24 spectrin-like repeats and four hinges. The N-terminal actin-binding domain and spectrin-like repeats 11–15 of dystrophin bind to costameric F-actin to aid in shock absorbance during muscle contraction. The CRD links dystrophin to the sarcolemma-bound β subunit of dystroglycan (113), which forms the dystroglycan complex. The extracellular α-dystroglycan links laminin-α2 along the sarcolemma. β-Dystroglycan associates with δ-sarcoglycan and participates in the stabilization of the sarcoglycan-sarcospan complex (62), which does not interact directly with dystrophin but strengthens the DAPC. The dystrophin CTD binds cytosolic proteins such as α-dystrobrevin 2 (16) and α- and β-syntrophins, which recruit sodium channels and signaling molecules, such as nNOS, via PDZ domains. Dystrophin interacts directly with nNOS via its spectrin-like repeats 16–17 (134). Dystrophin interacts indirectly with microtubules through ankyrin-B (13) and directly via spectrin-like repeats 20/23 (21, 185). The neuromuscular disorders associated with mutations in various members of the DAPC are specified. (*b*) Composition and structure of the utrophin-associated protein complex (UAPC), a complex similar to the DAPC that forms at the neuromuscular junction with utrophin instead of dystrophin. This complex acts as a receptor for laminin, agrin, and perlecan and links the actin cytoskeleton to the extracellular matrix via laminin-α4, -α5, and -β2 and only through the NTD of utrophin. The UAPC binds to rapsyn and is involved in the clustering of acetylcholine receptors. In addition, the utrophin CTD binds to MAST, which associates with microtubules, and nNOS can only be recruited indirectly through the syntrophins. Abbreviations used in the figure: AChR, acetylcholine receptor; BMD, Becker muscular dystrophy; CR, cysteine-rich domain; DMD, Duchenne muscular dystrophy; H1/H2, hinge 1/2; LGMD, limb-girdle muscular dystrophy; MAST, microtubule-associated serine/threonine kinase; MDC, merosin-deficient congenital muscular dystrophy; MFM, myofibrillar myopathy; nNOS, neuronal nitric oxide synthase; Syn, syntrophin. Adapted from Reference 64 with permission from Elsevier.

be commercially available, and be easily maintainable with low cost. To date, more than 60 dystrophin-deficient animal models have been described in the literature (156).

The *mdx* mouse is the most widely used DMD laboratory model (38). This mouse carries a point mutation in exon 23 of the *Dmd* gene that introduces a premature stop codon and results in the absence of full-length dystrophin expression. The *mdx* mouse presents a slightly shorter life span (52) and high plasma levels of creatine kinase (38) and develops a moderate, nonprogressive myopathy marked by early necrosis (91) and muscle degeneration followed by subsequent regeneration (155) without fibrosis (57). This model does not recapitulate all the gross symptoms and is only mildly affected compared with human DMD patients. Unlike skeletal and cardiac muscles, the diaphragm closely mimics the degeneration observed in DMD (211). The mild phenotype of the *mdx* mouse can be explained by the greater capacity of *mdx* muscle to regenerate compared with muscle from DMD patients and/or a compensatory function of utrophin. Thus, to exacerbate the phenotype, a series of *mdx* variants (61) and double-knockout animals have been developed. In particular, *dko* mice, which lack dystrophin and utrophin, present a much more severe phenotype that is comparable to human DMD (67). Recent *dko* strains such as *Cmah* (54) and *mdx/mTR* (194) mice exhibit severe phenotypes with symptoms that appear to closely reproduce the human condition.

Despite limitations resulting from body size, genetic background, incapacity to model the immune response to any gene therapy vectors, and pathological features, DMD murine models are, by their numerous advantages, essential for the establishment of therapeutic approaches. Furthermore, the availability of internationally accepted protocols for the *mdx* mouse [see the TREAT-NMD (Translational Research in Europe—Assessment and Treatment of Neuromuscular Diseases) website at **http://www.treat-nmd.eu**] is an invaluable resource in DMD research. Larcher et al. (136) recently described a promising *Dmd* mutated rat model with phenotypic properties close to the human DMD pathology. Rats are a convenient size and allow behavioral experiments and studies with high statistical power. Compared with *mdx* mice, these animals present an aggravated skeletal and cardiac muscle phenotype and represent a promising model to perform preclinical studies, especially on brain and heart.

In addition to the rodent models, several canine DMD models have been established (60, 205). The most extensively examined and characterized is the golden retriever muscular dystrophy dog (202), which presents a body size closer to humans, recapitulates many clinical features of human pathology (69), and mimics the immune response observed in DMD patients in gene therapy. These dogs are therefore useful for analyzing the mechanisms of the immune response and for scaling up human gene therapy, and they have been successfully used to study the therapeutic potential of different strategies, such as exon skipping (230) and dystrophin-independent therapy (49). However, in addition to the high cost of breeding, maintaining, and treating these dogs, their high degree of phenotypic variation makes it difficult to evaluate therapies. Although canine models have several advantages over *mdx* as an exon-skipping model, the disease-causing mutation lies outside of the region commonly affected in patients (5). Furthermore, for practical reasons, this model is never likely to supersede the *mdx* mouse in high-throughput studies.

Klymiuk et al. (123) recently generated a porcine model that lacks dystrophin. Pigs are phenotypically and phylogenetically closer to humans than either dogs or rodents are, and DMD pigs exhibit a progressive muscular dystrophy that is similar to human DMD but progresses more quickly. Porcine models have a number of practical advantages and present a size and physiology similar to those of humans.

Cats with hypertrophic feline muscular dystrophy exhibit a muscle pathology similar to that of the *mdx* mouse (237). Because their phenotype varies greatly from DMD patients and the expenses to maintain colonies are high, these animals are not widely used as DMD models.

Nonmammalian models of dystrophic pathology such as *dmDys Drosophila* (87), the nematode *Caenorhabditis elegans* (22), and the zebrafish (20) are interesting because of their potential for genetic manipulation. Easy breeding and maintenance make these models suitable for use in high-throughput studies. Although their musculature and phenotype do not recapitulate those of human DMD patients, these models are useful to provide other examples of a species-specific response to dystrophin deficiency and to screen small molecules that might allow normalization of skeletal muscle in response to dystrophin deficiency (116).

INFRASTRUCTURE

Clinical trials require a significant amount of infrastructure, which is often not in place for rare diseases. The TREAT-NMD network (**http://www.treat-nmd.eu**) has been instrumental in setting up much of the required infrastructure for DMD trials since 2007, including patient registries, a trial site registry, and standards of care (25, 188, 199). These tools are currently being maintained and are crucial when conducting multinational and/or multicenter clinical trials, which is more or less a given for rare diseases. Work is ongoing to collect natural history data and develop functional outcome measures for ambulant and nonambulant patients (179, 180). One should bear in mind that many of these tools were established while setting up clinical trials (see below). For example, the placebo arm of the phase 2b trial to test ataluren (~60 patients) provided the first natural history data for the 6-min walking distance (6MWD) test for DMD patients (44). The first results of large natural history studies evaluating many functional tests over time were reported only in 2013–2014, and several large natural history studies are still ongoing. Thus, most trials described below had to select patients based on suboptimal data. The lesson to be learned here for therapy development—not only for muscular dystrophies but for other rare diseases as well—is that natural history data and patient and trial site registries should ideally be in place before trials are initiated.

Outcome measures are a crucial part of both clinical trials and drug development. The 6MWD test is the most common primary end point in DMD trials. This test has been adopted from the cardiovascular field and was successfully used for the development of enzyme replacement therapy for another neuromuscular disorder (Pompe's disease). To obtain marketing authorization for a medication from the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA), the primary end point must be clinically meaningful (2). Because DMD is a progressive disease, over time the distance patients walk in 6 min levels off around the age of eight years and then declines (180). The likelihood that patients lose ambulation in the next year rapidly increases once patients walk less than 350 m in 6 min. As such, the 6MWD is a readout for whether a treatment slows disease progression. However, the clinical benefit, i.e., slower disease progression, is reflected not only by treated patients walking (for example) 30 m more compared with untreated patients, but also by prolonged ambulation, delayed requirement for assisted ventilation, and so on. The disadvantage of the 6MWD test is that it is useless for nonambulant patients. Upper limb function tests are in development for this group and will hopefully be implemented in future trials.

Another disadvantage of any functional test for therapies that slow down disease (i.e., all therapies currently in development) is that DMD progression is not that rapid, and therefore trials must be long to show a significant clinical benefit. Ongoing development has focused on developing surrogate end points or biomarkers that can detect therapeutic effects earlier, enabling shorter trials—for example, the use of magnetic resonance imaging to assess muscle quality, or measurement of molecular biomarkers such as serum proteins and microRNAs (10, 175, 243). Notably, because these markers can be used only when they correlate with clinical benefit (i.e., a functional

outcome measure used as a primary end point), they require extensive validation after their discovery. Developing potential biomarkers is therefore a lengthy process that involves test optimization and standardization to fulfill regulatory requirements (3). There is an ongoing need for candidate biomarkers, and it is good to see that recent research has uncovered potential markers in serum and urine (see **Supplemental Table 1**; follow the **Supplemental Materials link** from the Annual Reviews home page at **http://www.annualreviews.org**). Other types of biomarkers under development include genetic modifiers, pharmacodynamic biomarkers, and magnetic resonance imaging (3, 10, 78) (**Supplemental Table 1**).

Supplemental Material

DYSTROPHIN-BASED THERAPY

Even before the discovery of dystrophin, cell therapy was evaluated as a therapeutic approach for DMD, on the reasoning that injecting cells isolated from a healthy individual would deliver a functional copy of whatever gene was deleted and allow repair of damaged muscle fibers (181). Myoblasts (muscle stem cells) can be cultured ex vivo to obtain enough cells for transplantation. Unfortunately, in studies of this approach, the vast majority of myoblasts did not migrate into damaged muscle but died shortly after injection (76); even after local injection, migration is very limited (208). High-density injections (100 in an area of 1 cm^2 or 0.25 cm^2) increased dystrophin positive fibers somewhat (207), but this approach is suitable only for superficial muscles.

As an alternative, investigators have searched for other stem cells migrating from the bloodstream into muscle. These have been identified from blood, vessel walls, fat, and bone (165), and induced pluripotent stem cells were recently targeted into the myogenic lineage (106). Safety trials have been performed or are ongoing for $CD133^+$ cells and mesangioblasts (222; G. Cossu, personal communication). Yet the percentage of cells migrating into muscle is generally <1%—extremely low, considering that muscle constitutes 30–40% of human body mass. It is challenging to culture large amounts of these stem cells (221). Moreover, these cells tend to differentiate in vitro and can be maintained only for a limited number of passages. A challenge often overlooked is that stem cells that do reach muscle arrive not into a healthy muscle but rather into fibrotic and chronically inflamed tissue, producing many factors opposing regeneration (90). It is unlikely that a few healthy stem cells can revert this. Rather, upon receiving signals from existing dystrophic muscles, they may transdifferentiate into fibrotic tissue. Current DMD stem cell therapy studies focus on optimizing isolation, culture, and delivery and improving muscle quality and create a less hostile environment.

After dystrophin's discovery, and sparked by other early gene therapy efforts, bold claims were made that dystrophin gene therapy would be possible within ten years. It soon became clear that gene therapy was not as simple as initially assumed. First, the *DMD* gene is exceptionally long (2.2 Mb), and even the cDNA (11 kb) is well beyond the capacity of most viral vectors (187). Second, muscle is postmitotic, and individual fibers, fiber bundles, and muscles are all surrounded by layers of connective tissue that shield their content from large viruses and macromolecules such as plasmids. Little headway was made using adenoviral vectors, and even direct intramuscular injection of dystrophin cDNA only marginally restored dystrophin expression (189). Although the tiny adeno-associated virus (AAV) efficiently infected myofibers, its capacity (4.5 kb) precluded uptake of full-size dystrophin cDNA (88).

Salvation came from the finding that a few very mildly affected Becker muscular dystrophy patients had dystrophins with substantial internal truncations of up to 46% (71). The removal of less essential domains enabled the development of minidystrophins, which generally contain two actin-binding domains, two or three hinge regions, approximately five spectrin repeats, and a cysteine-rich region (11). The corresponding cDNA fits into AAV, and these constructs improved

muscle quality and function in the *mdx* mouse model (11). Because these engineered dystrophins are much smaller than Becker dystrophins, how functional they will be in humans remains uncertain, and the extensive reconstruction may cause immunogenicity resulting from new epitopes. In patients, direct injection of minidystrophin AAV resulted in low levels of local dystrophin restoration for only two of six patients, whereas an immune response to AAV was observed for all patients (32).

Besides delivery issues, virus-based gene therapy for humans, like cell therapy, involves serious upscaling. AAV production has been significantly improved with the implementation of the baculovirus system, which allows GMP production at a larger scale (130). For delivery, high-pressure limb perfusion has been developed in dogs and monkeys (55, 137). This technique shows good transduction of muscles in the treated limb, and for treating a lower limb, an infusion with saline of 20% of the limb volume was tolerable in human adults (77). A key remaining challenge for AAV gene therapy is the immune response against the vector, which precludes recurrent treatment (necessary because currently treatment would be on a limb-by-limb basis) and possibly also the application of next-generation viral vectors in previously treated patients.

Besides gene addition, mutations can also be corrected by gene editing through the cell's own mismatch repair mechanism. Until recently, this was achieved with meganucleases or zinc finger proteins (192), which did not allow much flexibility. However, with the development of transcription activator–like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) systems, genome editing has become much more straightforward. Different gene correction modes have been followed using cultured cells and induced pluripotent stem cells derived from DMD patients, from traditional gene correction for small mutations (using a template with the correct sequence) to the introduction of small mutations at splice sites that cause exon skipping (see below) to complete removal of exons using nucleases (142, 178). The key challenge for genome editing remains low efficiency: Generally, only 1 in 100–1,000 cells is successfully targeted. In vitro, one can select the targeted cells and further expand them. However, for this ex vivo genome editing, the subsequent delivery has the same hurdles as classical cell therapy. For in vivo application, the current bottleneck is delivery of the TALENs or CRISPR/Cas nucleases (and optionally a template) to a sufficient number of myonuclei.

Given the challenge of delivering cells or genes to muscle, research has also focused on smaller molecules that are easier to deliver. Antisense-mediated exon skipping uses modified, complementary RNA or DNA antisense oligonucleotides (AONs) to target exons in the pre-mRNA, preventing their recognition and inclusion in the mRNA by the splicing system (128). When the proper exons are targeted, this approach can restore the reading frame or bypass mutations in in-frame exons, thus allowing the patient's own gene expression to produce Becker-like dystrophins. To improve stability and target recognition or alter biochemical properties, AONs can be chemically modified in many ways (114).

A proof of concept for this approach in patient-derived cell cultures and the *mdx* mouse model was achieved by the early 2000s (151, 223). Around 2005, the first open-label phase 1 and 2 clinical trials were initiated using local and systemic injection of AONs based on 2-*O*-methyl-phosphothioate (2OMePS) (224) and morpholino-phosphorodiamidate oligomer (PMO) (120) chemistry, targeting the skipping of exon 51, which if successful would benefit 13% of all DMD patients. Some dystrophin protein was produced in all trials. The longest open-label extension trial ran from 2009 to 2013 in 12 patients with the 2OMePS chemistry (drisapersen) and showed maintenance of the 6MWD in 8 out of 10 ambulant patients (84). At the end, patient ages ranged up to 16 years, i.e., well beyond the typical age of ambulation decline or loss. In parallel, several randomized, placebo-controlled phase 2 trials were initiated. In cohorts of early-stage patients

(ages 6–8 years), improved 6MWD was observed for treated patients compared with those receiving a placebo (229). An ongoing phase 2 trial for the PMO chemistry (eteplirsen) suggests slower disease progression than natural history, based on 6MWD data for up to 168 weeks (161), and several larger phase 2 and 3 trials have recently been initiated for eteplirsen. A very large (186 patients), 2:1 placebo-controlled phase 3 trial ran from 2011 to 2013 for drisapersen, but it failed to meet its primary end point of a significant improvement in 6MWD after 48 weeks of treatment compared with placebo. Subsequent data analysis showed that the average patient age was over one year older than patients in phase 2 trials (i.e., closer to the onset of decline), resulting in a poorer condition. This was corroborated by the finding that phase 3 patients had a lower baseline 6MWD. Moreover, for 80 patients who received open-label extension treatment in year two, the results were indicative of a treatment effect, suggesting that more advanced patients needed longer treatment for an effect to take hold. Currently, based on the phase 2 and 3 data, filings are under way for drisapersen marketing authorization, in close consultation with the FDA and EMA.

A challenge of AON-based skipping remains the need for repeated treatment because of turnover of AONs, transcripts, and protein. A more permanent form of exon skipping makes use of modified small nuclear ribonucleoproteins in which the original antisense moiety is replaced by an antisense sequence targeting the exon of choice. AAV-mediated delivery of these ribonucleoproteins showed dystrophin production in mouse and dog models (24, 86). Although in principle more permanent, this approach clearly faces the immunological and delivery challenges of regular gene therapy.

Future developments in exon skipping will relate to chemistry and application scope. Current chemistries do not achieve complete exon skipping and dystrophin restoration. New chemistries have shown promising results, with increased efficiency in skeletal muscles and especially the heart (23, 85). However, the tolerability and safety for these new chemistries need to be assessed in humans, especially as patients need to be treated chronically. Although the chemistries currently tested in trials are tolerated by humans, there are side effects. Proteinuria has been reported for nearly all patients treated with 2OMePS AONs and some patients treated with PMO AONs. Proteinuria was reversible during treatment breaks. Furthermore, for 2OMePS, thrombocytopenia has been observed in some patients, and injection site reactions were observed in the majority after subcutaneous injections (84, 161, 229).

Skipping is intrinsically exon specific, so AONs benefit only a subset of patients (4). Although drisapersen and eteplirsen both target exon 51 and apply to 13% of patients, further trials are ongoing or planned for exons 44, 45, and 53. Jointly, these would benefit ~38% of DMD patients (26). Hopefully a faster trajectory for clinical development can be adopted should marketing authorization be obtained for initial AONs (2).

Finally, patients carrying nonsense mutations should benefit from antibiotic analogs inducing stop codon read-through. The first compound tested in DMD patients was gentamicin (150), which resulted in significantly lower serum creatine kinase levels (a marker for muscle damage) in patients with nonsense mutations but not in patients with deletions. Furthermore, a subset of nonsense mutation patients showed some dystrophin restoration. Unfortunately, owing to the risk of renal failure and irreversible ototoxicity, chronic use of gentamicin is impossible. High-throughput screening for compounds with nonsense read-through potential yielded PTC124 (ataluren or Translarna). This compound resulted in some dystrophin restoration in the *mdx* mouse (236) and also restored protein deficiencies in numerous other cell and animal models carrying nonsense mutations in other genes (98). Ataluren can be orally delivered and is well tolerated by healthy volunteers and DMD patients. A small dose-finding phase 2 trial showed a very mild increase in dystrophin expression in two-thirds of patients (79). Subsequently, a large one-year clinical trial failed to meet its primary 6MWD end point (44). Notably, patients receiving a low

dose of the drug showed an almost significant effect, whereas patients on the higher dose showed results identical to the placebo group. It was then found that the ataluren response is bell shaped, with the low dose being more optimal. Post hoc analysis showed a significant effect for patients treated with the low dose (44). Based on these insights, conditional marketing authorization was awarded by the EMA in August 2014, making ataluren the first licensed drug for DMD (44), although the company still needs to provide additional evidence for clinical benefit in a phase 3 clinical trial currently under way.

Restoring the missing protein in a progressive disease like DMD will not cure it but rather will slow it down (for a recent review, see Reference 1). Enhancing dystrophin expression relies on muscle quality because dystrophin is expressed only by muscle fibers and not by fibrotic or adipose tissue. The muscles of DMD patients undergo fibrosis and adiposis from early on and may continue to do so, depending on the age at the start of treatment. The therapeutic effect relies on muscle quality at the initiation of treatment, and this requirement will apply equally to other gene and cell therapies and even to therapies for other muscular diseases. This necessitates the development of parallel, dystrophin-independent therapies that improve overall muscle condition.

DYSTROPHIN-INDEPENDENT STRATEGIES

This section focuses on the genetic strategies related to correcting or compensating for the defect. Numerous pharmacological strategies to slow down the disease process, such as those reducing inflammation or fibrosis, are in development as well, and these have been reviewed previously (75). The advantage of a compensatory approach is that it is applicable to all patients independent of the type of mutation while avoiding an immune response such as that observed following minidystrophin gene replacement (159).

Utrophin Modulation

Utrophin is a structural and functional autosomal paralog of dystrophin (147). This large cytoskeletal protein (394 kDa) shares 80% of sequence homology with dystrophin, and the primary sequence suggests functional redundancy (27, 147, 216). Unlike dystrophin, utrophin is ubiquitously expressed and is found at the sarcolemma in early human development (184, 201). In adult skeletal muscle, it is localized at the neuromuscular and myotendinous junctions (177) and at the sarcolemma in regenerated myofibers (102). In the *mdx* mouse, the level of utrophin is naturally increased (1.8-fold) and is independent of regeneration (235), which may be due to the stabilization of utrophin protein in the absence of dystrophin at the sarcolemma. The mild phenotype observed in the *mdx* mouse has been proposed to result from the efficient regeneration process in this model, but it may also be that there is some compensation by utrophin. This view is supported by the fact that mice deficient in both dystrophin and utrophin show a progressive muscular dystrophy very similar to that seen in DMD patients (68). Proof of principle that utrophin can compensate for the absence of dystrophin comes from extensive studies establishing that an increase of utrophin protein levels in transgenic *mdx* mice prevents pathology that is dependent on the amount of utrophin expressed (217). Modulation of the utrophin level results in the formation of the utrophin-associated protein complex (an alternative to the DAPC) and significantly improves sarcolemmal stability (217, 219). Importantly, this level of utrophin increase is not toxic if expressed in all tissues (80) and is significantly less than the levels of utrophin found in normal kidney and liver (217). Furthermore, induction of utrophin at birth also prevents pathology (210). The earlier the administration of utrophin is, the better the outcome is, a result also found in studies using dystrophin transgenes (115).

Utrophin does not restore nNOS localization (141) and exhibits modes of interaction with actin (51) and microtubules (21) that differ from those of dystrophin, and it therefore may not prevent microtubule lattice derangement. However, because utrophin can replace dystrophin in the *mdx* model, the microtubule arrangement may not be the sole contributing factor to a more complex mechanism of contraction-induced injury. Furthermore, very mildly affected Becker muscular dystrophy patients lack the nNOS binding site (71), suggesting that there may be compensatory nNOS pathways.

Utrophin is expressed from at least two promoters, known as promoters A and B (43). Expression from promoter B is predominantly in endothelial cells and is the isoform seen in blood vessels, whereas expression from promoter A occurs in muscle and other tissues (234). By modulating the utrophin A promoter, several small molecules—including heregulin (131), nabumetone (173), and L-arginine (228)—are able to upregulate the utrophin-encoding gene. The first small-molecule drug in development designed to increase and maintain utrophin transcript levels, SMT C1100, has started clinical trials (220). Preclinically, this orally bioavailable 2-arylbenzoxazole derivative (53) prevents the pathology in the *mdx* mouse (218). Encouraging results from a phase 1 healthy volunteer study completed by Summit Therapeutics showed that SMT C1100 is safe and well tolerated (220), with plasma levels achieved above those believed to be required to modulate utrophin (220). Clinical trials of SMT C1100 in boys with DMD are ongoing to confirm appropriate levels of exposure (ClinicalTrials.gov identifier NCT02383511). Preclinical studies of the compounds chemically related to SMT C1100 suggest that these second-generation molecules lead to increased utrophin expression in skeletal, respiratory, and cardiac muscles, resulting in improved sarcolemmal stability (93). These results endorse the therapeutic potential of utrophin modulation as a disease-modifying therapeutic strategy for all DMD patients irrespective of their dystrophin mutation.

Muscle structure influences utrophin expression in the *mdx* mouse (17, 144). In slow/oxidative muscle, utrophin levels are elevated (50) and may contribute to enhancing the resistance of slow muscle fibers to contraction-induced damage in DMD (59, 169, 232). The promotion of the slow/oxidative myogenic program by modulating peroxisome proliferator–activated receptor gamma coactivator 1-alpha (PGC-1α) or the downstream effectors results in a 1.5–2-fold increase in utrophin mRNA levels and mitigates the dystrophic phenotype (reviewed in 75). These results indicated a promoter-based synergism (8, 168) and that a multitargeting utrophin strategy is feasible. A more promising approach is the administration of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a nucleoside that activates PGC-1α and peroxisomal proliferator–activated receptor β/δ (PPARβ/δ) via AMP-activated protein kinase (AMPK), a nexus of skeletal muscle plasticity (145). AICAR treatment in the *mdx* mouse results in elevated levels of sarcolemmal utrophin (2.1-fold) and β-dystroglycan protein in fast muscles but not in slow muscles via a fast-to-slow muscle fiber transition (146).

Direct utrophin protein replacement is one other attractive strategy (46). Sonnemann et al. (209) used the TAT protein transduction domain of the human immunodeficiency virus (HIV-1) to create chimeric proteins deliverable by microutrophin (TAT-μUtr) and utrophin protein (TAT-Utr). Preclinical studies in *mdx* and *dko* mice looked promising, but further progress has not been reported. Biglycan, a small leucine-rich proteoglycan that is important for the maintenance of muscle cell integrity, is highly expressed in the extracellular matrix of DMD and *mdx* skeletal muscle (31, 147, 244) and is abundant in regenerating muscle (48). It participates in the transcription and structural regulation of multiple components of the DAPC, notably nNOS (164), and is able to recruit utrophin to the sarcolemma (7). A single systemic dose of recombinant human biglycan in two- to three-week-old *mdx* mice resulted in functional improvement. As this approach did not ameliorate the pathology in the *dko* mice, the benefits of recombinant human

biglycan were related to a 1.5-fold increase in utrophin protein expression without any change at the transcriptional level, suggesting that functional benefits arise from posttranscriptional effects.

α7-Integrin Upregulation/Laminin-111

Integrin/laminin complexes serve as mechanosignaling anchors, bind laminin, and link the extracellular matrix with the intracellular cytoskeleton (108). In particular, α7β1-integrin/laminin-211 plays an essential role in the functional integrity and maintenance of skeletal myoblasts and adult myofibers (103, 154). Defects in the components of this complex cause muscular dystrophy, illustrating the essential role of the α7 chain (103, 95). The α7β1-integrin/laminin-211 complex shares structural and signaling roles and presents some functional redundancies with the DAPC (190, 191). Interestingly, α7-integrin expression is increased at the sarcolemma in the *mdx* mouse and DMD patients (103), suggesting that integrin upregulation may functionally compensate for the absence of dystrophin. Transgenic overexpression of α7-integrin in *dko* mice alleviates pathology, extends viability and mobility, and reduces kyphosis (40, 41). The enhancement of α7-integrin levels mediates sarcolemmal stability and promotes proliferation, adherence, and activation of satellite cells, resulting in improved regeneration (41, 143) and a significant functional improvement (101). Favorably, eightfold overexpression of α7-integrin does not demonstrate visible toxicity or disruption to global gene expression profiles (143). Thus, similar to utrophin, small-compound screening for α7-integrin modulators such as valproic acid has been developed (97). However, the potential toxicity of valproic acid must be carefully assessed (135).

Another candidate of interest is laminin-111 (190). Injection of laminin-111 protein in the *mdx* mouse increased expression of α7-integrin, improved skeletal muscle stem cell function and regenerative capacity, stabilized the sarcolemma, and protected muscle from exercise-induced damage (190, 191). However, a different study with transgenic expression of the laminin α1 chain to enhance heterotrimer formation of laminin-111 in the *mdx* mouse reported no improvement of the dystrophic symptoms (83), indicating that further studies are required to verify the functionality of laminin-111 protein therapy in DMD.

Myostatin, Follistatin, and Other Muscle Growth Strategies

Myostatin, a negative regulator of muscle growth (158), has drawn intense interest in the development of strategies to inhibit the activity of this factor in muscle-wasting-related disorders. Myostatin blockade by any of several agents—such as myostatin antibodies (28), myostatin propeptides (18, 214), the active form of mutated myostatin (206), small leucine-rich proteoglycans such as decorin (94), or activin receptor IIB (139)—ameliorates the pathology in the *mdx* mouse. Nevertheless, clinical trials in humans have been disappointing because of a lack of improvement in muscle strength (231) or adverse effects (12). Despite these disappointing results, other clinical trials based on blocking myostatin activity have started (174, 183).

A more potent factor is follistatin. Indeed, myostatin-deficient mice carrying a follistatin transgene possess approximately four times the muscle mass of wild-type mice, demonstrating the existence of other regulators of muscle mass with activity similar to that of myostatin (138). Based on successful preclinical studies in the *mdx* mouse (99) and nonhuman primates (129), this approach has recently been validated in a phase 1/2a clinical trial in Becker muscular dystrophy patients. There were no adverse effects encountered after treatment, and encouraging histological benefits and a longer distance walked in the 6MWD test were noted (162).

Other pathways and treatment strategies aiming to increase muscle fiber size and strength are under investigation for DMD. Strategies centered on insulin-like growth factor I (19, 203) or

the bone morphogenetic protein (BMP) pathway (196, 197) have been described and recently reviewed.

Phosphodiesterase Signaling Pathways

During muscular contraction, nNOS, the predominant source of NO, is required to equilibrate muscle oxygenation and protect the muscle against excessive sympathetic vasoconstriction. In DMD patients and the *mdx* mouse, dystrophin deficiency and the loss of α-syntrophin lead to mislocalization of nNOS at the sarcolemma and a significant reduction of this muscle-specific splice variant of nNOS (36, 134). As a result, unlike healthy children, in whom normal nNOS confers maintenance of blood flow, DMD patients suffer from functional muscle ischemia (195, 215). Repeated bouts of functional ischemia could be an important contributory factor in DMD muscle damage, and boosting NO signaling in order to circumvent this ischemia is a promising field of investigation. Multiple strategies—including transgenic expression of nNOS (233) or dystrophin minigenes that restore nNOSμ at the sarcolemma (134), treatment with L-arginine (228), and treatment with NO-donating drugs (37, 198)—have been developed and show histological and functional improvements in the *mdx* mouse. More recently, phosphodiesterase 5A (PDE5A) inhibitors such as sildenafil or tadalafil, which prevent the degradation of the guanosine 3′,5′-monophosphate cGMP, the downstream target of NO in vascular smooth muscle, were shown to rescue dystrophic skeletal muscle and prolong survival in dystrophin-deficient zebrafish (116) and to alleviate muscle ischemia and improve cardiac dynamics in *mdx* mice (6, 118). These preclinical results support PDE5A inhibitors for clinical trials, especially as sildenafil (Viagra, Revatio) and tadalafil (Cialis) are currently available for clinical use.

Clinical studies in Becker muscular dystrophy (152) and DMD patients (176) have reported encouraging results, although another trial testing sildenafil in DMD patients was prematurely terminated by the safety committee because of potential detrimental effects on heart function (140). Tadalafil has more optimal pharmacokinetic characteristics for chronic use, and a large trial involving >300 DMD patients is currently being conducted by Eli Lilly to assess its effects.

Steroids

Steroids such as prednisone and deflazacort are the current standard of care for DMD (45). They prolong ambulation and modestly improve muscle strength, cardiopulmonary function, and functional outcome in DMD (112, 160). Nevertheless, long-term corticosteroid treatment has significant side effects, including weight gain, short stature, puberty delay, behavioral issues, and pathologic bone fractures (58). These defects prompted the use of many different doses, types of steroids, and regimens (89), and many boys with DMD are undertreated or overtreated. A promising oral glucocorticosteroid analog named VBP-15 was recently shown to improve muscle strength without side effects in the *mdx* mouse (65, 100), and a phase 1a clinical trial is currently under way on human healthy volunteers.

FINAL PERSPECTIVE

The identification of the dystrophin gene as the cause of DMD and Becker muscular dystrophy has led to improved diagnosis of the disorders and unique insight into the biochemistry of skeletal and cardiac muscle. It has also led to rational approaches to therapy for these disorders and clinical trials of some therapies, and these approaches set an example for research in other disorders. We have learned much over the years, yet much remains to be learned before therapies are corrective of the pathology seen in the affected individuals.

DISCLOSURE STATEMENT

A.A.-R. and G.-J.B.v.O. are employed by the Leiden University Medical Center, which has patents on exon skipping technology that have been licensed to Prosensa; A.A.-R. and G.-J.B.v.O. are coinventors on some of these patents and are entitled to a share of potential royalties. A.A.-R. serves as an ad hoc consultant for Guidepoint Global, GLC, and PTC Therapeutics, remuneration for which is paid to the Leiden University Medical Center. K.E.D. and G.-J.B.v.O. are on the Science Advisory Board of Prosensa/BioMarin, and K.E.D. and L.M.K. are on the Science Advisory Board of Summit Therapeutics. K.E.D. is a shareholder of Summit Therapeutics. L.M.K. has a research grant from Pfizer.

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