Molecular Mechanisms and Treatment Options for Muscle Wasting Diseases

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Abstract

Loss of muscle mass can be the consequence of pathological changes, as observed in muscular dystrophies; or it can be secondary to cachexia-inducing diseases that cause muscle atrophy, such as cancer, heart disease, or chronic obstructive pulmonary disease; or it can be a consequence of aging or simple disuse. Although muscular dystrophies are rare, muscle loss affects millions of people worldwide. We discuss the molecular mechanisms involved in muscular dystrophy and in muscle atrophy and present current strategies aimed at ameliorating these diseases. Finally, we discuss whether lessons learned from studying muscular dystrophies will also be helpful for halting muscle loss secondary to nondystrophic diseases and whether strategies to halt muscle atrophy have potential for the treatment of muscular dystrophies.

INTRODUCTION

The muscular dystrophies comprise a group of inherited disorders that occur as a consequence of multiple genetic mutations, inducing muscle degeneration and weakness. In the more severe dystrophies, such as Duchenne muscular dystrophy (DMD), the loss of muscle integrity leads to an inability to ambulate and eventually leads to death due to respiratory failure (as a result of diaphragmatic muscle failure). Muscle loss also occurs as a consequence of rather frequent, noninherited conditions. Examples include cachexia, which can be a secondary consequence of cancer, AIDS, chronic obstructive pulmonary disease (COPD), renal disease or sepsis, and sarcopenia, which is the loss of muscle that occurs as a consequence of aging. In this review, we discuss the molecular mechanisms that cause muscular dystrophies and those that control muscle size and muscle maintenance in genetically normal muscle. We also present some of the most advanced experimental treatment strategies for muscular dystrophies and discuss whether pathways involved in the control of muscle size could eventually also be targets for the treatment of muscular dystrophies.

MUSCULAR DYSTROPHIES

The first gene discovered that causes the most prevalent muscular dystrophy, affecting 1 out of 3,500 boys, was *dystrophin* (1). The discovery of *dystrophin* was among the first studies that successfully linked the mutation of a gene to a particular disease. The *dystrophin* gene is localized on the X chromosome (position Xp21.2) and has a total size of 2.5 Mb, which makes it the largest gene in the human genome (encoding 79 exons). Mutations in *dystrophin* cause DMD or Becker muscular dystrophy (BMD), which is a milder allelic form of the disease. The reason for the milder disease course in BMD is residual expression of a truncated form of dystrophin. As a consequence, the symptoms are substantially alleviated, which in rare cases allows the patients to experience an almost normal life (e.g., 2, 3). Although the majority of *dystrophin* mutations are deletions or duplications, even single point mutations have been reported. Moreover, there is no particular hotspot for mutations in the *dystrophin* gene. Approximately one-third of DMD cases are due to novel germ-line mutations; the rest are inherited from the maternal line. For these reasons, it is difficult to establish routine prenatal diagnostics for the *dystrophin* gene, indicating that the number of patients will remain stable in the future.

Besides the most prevalent disease, DMD, more than 30 additional genes have been linked to different types of muscular dystrophies. They are all characterized in their late stages by the loss of muscle, due to muscle fiber degeneration, with a coincident appearance of fibrosis. A common feature of most muscular dystrophies is the attempt of muscle to compensate for the loss of muscle fibers due to apoptosis or necrosis by enhancing regeneration. Thus, initial signs of muscular dystrophies are repeated cycles of muscle degeneration and regeneration, which are histologically characterized by the presence of centralized myonuclei and an increase of muscle-specific cytosolic proteins, such as creatine kinase, in the circulating blood.

Although the detailed mechanisms underlying muscular dystrophies are still not known, the past two decades have witnessed tremendous progress in the deciphering of the pathways involved. Despite the fact that more than 30 genes have been implicated in muscular dystrophies, recent evidence suggests that only a few common pathways are affected in most of these diseases. Before molecular diagnostics were available, muscular dystrophies were subdivided into different groups based on phenotype and time of onset. Such subgroups are, for example, congenital muscular dystrophies, dystrophinopathies, limb-girdle muscular dystrophies, myotonic dystrophy, or Emery-Dreifuss muscular dystrophy (reviewed in Reference 4). Although the overall clinical

picture can significantly differ between these disease groups, thanks to the advances in genetic profiling it has been shown that different mutations in the same gene can cause diseases belonging to different subgroups. For example, mutations in fukutin-related protein (FKRP), a putative glycosyltransferase that is implicated in the glycosylation of α-dystroglycan, can cause a very severe form of a congenital muscular dystrophy (5), but is also responsible for a rather mild form of limb-girdle muscular dystrophy (6). Thus, the current diagnostic procedure for patients suffering from a muscular dystrophy often also includes the mapping of the mutation.

The products of mutated genes that cause muscular dystrophies can be localized in all cell compartments, including the basement membrane, the sarcolemma, the sarcoplasm, and even the nucleus. Despite the different locations of these gene products, current knowledge predicts that most of the affected genes are functionally interconnected (**Figure 1**). For example, recent evidence

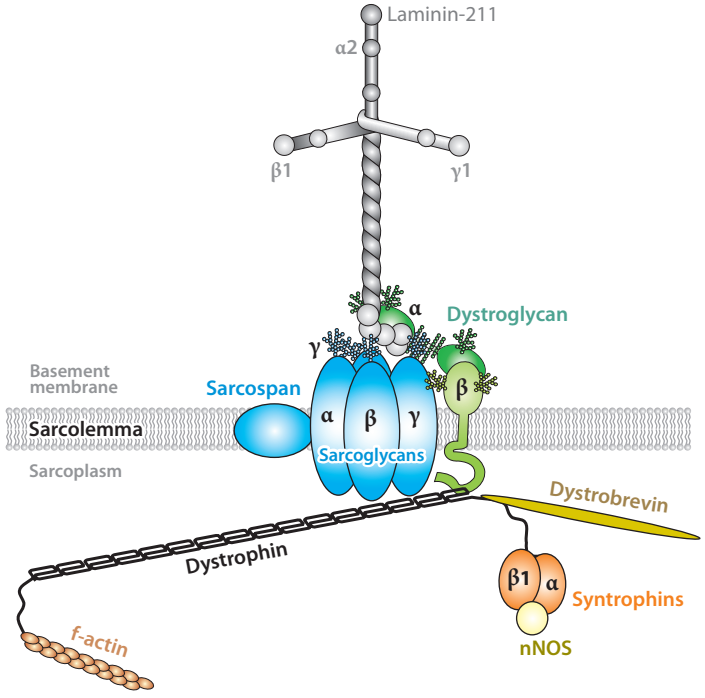


Figure 1

The dystrophin glycoprotein complex. This protein complex includes the peripheral membrane protein α-dystroglycan (α) and the transmembrane protein β-dystroglycan (β), which originate from posttranslational cleavage of its precursor dystroglycan. The peripheral membrane component α-dystroglycan binds to the basement membrane component laminin-211 via its carbohydrate moiety. Within the sarcolemma, β-dystroglycan associates with the sarcoglycans composed of α, β, δ, and γ subunits and sarcospan. In the sarcoplasm, β-dystroglycan binds to the C-terminal part of dystrophin. Dystrophin also binds to dystrobrevin, and α- and β1-syntrophin, which in turn associate with neuronal nitric oxide synthase (nNOS). With its N-terminal end, dystrophin binds to the f-actin cytoskeleton. The middle part of dystrophin consists of rod-like domains. Mutations in genes coding for components of the dystrophin glycoprotein complex lead to muscle dystrophies. For example, mutation of *LAMA2*, encoding the α2 chain of laminin-211, causes the congenital muscular dystrophy MDC1A; mutations in sarcoglycans cause limb-girdle muscular dystrophies; changes in the glycosylation pattern of α-dystroglycan cause different forms of congenital muscular dystrophies; and mutations in *dystrophin* cause Duchenne or Becker muscular dystrophy. The data indicate that the dystrophin glycoprotein complex is important to link basement membrane to the f-actin cytoskeleton.

from congenital muscular dystrophies, which are severe forms of the disease with an early onset, indicates that mutations in putative glycosyltransferases, localized in the endoplasmic reticulum or the Golgi apparatus, affect the glycosylation of the peripheral membrane protein α-dystroglycan (7). In skeletal muscle, the major ligand of α-dystroglycan is laminin-211, and its binding depends on posttranslational modification (8). Similarly, mutations in sarcoglycans cause different types of limb-girdle muscular dystrophies (9). Thus, studies focusing on molecular mechanisms have led to the intriguing possibility that the primary cause of most muscular dystrophies is the impairment of the connection between the basement membrane and the cytoskeleton.

Molecular characterization of the genes that are mutated in muscular dystrophy has shown that most of their protein products form a multiprotein complex, named the dystrophin-glycoprotein complex (**Figure 1**). This complex is thought to form a physical connection of the basement membrane to the cytoskeleton that confers physical stability to muscle fibers during contraction. Inability to withstand these forces would result in the detachment of muscle fibers from the basement membrane, resulting in the generation of small tears in the sarcolemma or the detachment of muscle fibers from the f-actin cytoskeleton. The consequence of this damage to the sarcolemma is the unregulated influx of calcium into muscle fibers and subsequent triggering of several downstream pathways, including activation of calcium-dependent proteases, which eventually lead to muscle fiber necrosis. An example of such calcium-activated proteases are the calpains (reviewed in Reference 10). Necrosis of the muscle fiber after injury allows cytosolic enzymes to be released into the bloodstream. As mentioned above, high levels of creatine kinase in the blood and a high percentage of muscle fibers with centralized myonuclei are both hallmarks of many muscular dystrophies. There is also evidence that this leakage of calcium into muscle fibers triggers pathways that are known to be involved in muscle atrophy (see Molecular Mechanisms of Muscle Atrophy and Hypertrophy, below).

The degeneration of muscle fibers is followed by the activation of satellite cells in an attempt to regenerate the skeletal muscle. Although muscle regeneration is highly efficient in normal individuals—and in wild-type mice—several lines of evidence indicate that regeneration is inefficient in muscular dystrophy patients and mouse models thereof. For example, regeneration is impaired in *mdx* mice (11, 12), a dystrophin-deficient mouse model of DMD (13). The frequent cycles of muscle fiber degeneration and regeneration are also thought to deplete the pool of satellite cells in dystrophic muscle. Thus, in a late stage of the disease, the skeletal muscle compartment is occupied by nonmuscle cells, such as adipocytes, macrophages, or fibroblasts. Thus, replenishment of the satellite cell pool might be a promising strategy in the treatment of muscular dystrophies (reviewed in Reference 14).

APPROACHES TO THERAPY IN MUSCULAR DYSTROPHIES

Currently, there is no curative treatment for any muscular dystrophies. For many reasons, one being the relatively large number of patients, DMD is probably the most studied and best understood muscular dystrophy. The improved understanding of mechanisms underlying the disease has resulted in several proposed treatments, most of which have shown some efficacy in animal models of DMD. Among the many palliative treatments, the use of glucocorticoids has been demonstrated in several studies to slow the decline of muscle strength, to prolong ambulation, and to support respiration (15). Based on the strong clinical evidence, glucocorticoids are now the standard of care for the treatment of DMD patients (16). Other treatments for which there have been claims of some efficacy based on animal studies and small clinical trials include food supplements, such as creatine, coenzyme Q10, or antioxidants. However, none of these have been shown to improve muscle strength or disease outcomes such as survival when tested in a rigorous clinical trial

(e.g., 17) although there have been occasional reports of improvement in some patients (18). As most food supplements are well tolerated, the decision to take those additives remains with the patient, but their use is not generally recommended.

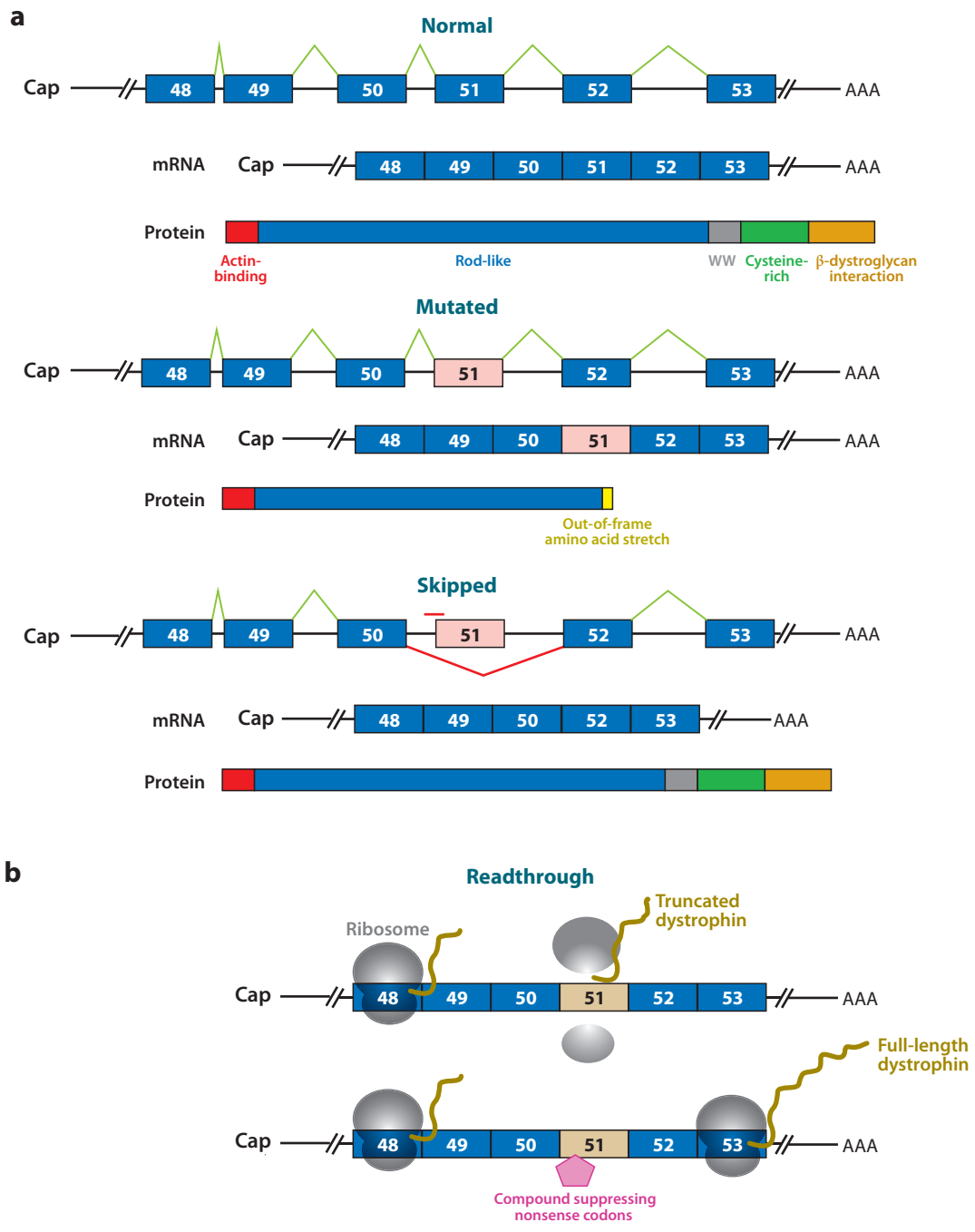
Targeting the Disease-Causing Gene

Muscular dystrophies are all genetic in origin; thus, once the mutation causing the disease is known, the most straightforward approach to therapy would be to reintroduce a wild-type, or normal, copy of the mutated gene. Indeed, the discovery of *dystrophin* twenty years ago triggered high hopes that a therapy for DMD would soon be available. However, the development of gene therapy approaches has so far not been successful, mainly because of problems with delivery of an additional, unmutated copy of the gene to the large volume of skeletal muscle that exists in the human body. Also complicating matters are some recently described adverse events with gene therapy clinical trials—such as the death of a patient after injection of adeno-viral particles (19) or the development of leukemia in children after retrovirus-mediated gene transfer (20). Despite these major setbacks, there have been advances in the field of gene therapy, and there is still hope that viral vectors will become of use in the treatment of muscular dystrophies (21). In particular, the use of adeno-associated virus (AAV) shows promise because it has a rather low immunogenicity and can home to skeletal muscle after intravenous injection (22). The big disadvantage of AAVs, however, is the low packaging capacity of only 5 kb, which makes it necessary to generate artificial, miniaturized forms of large genes such as *dystrophin* (full-length cDNA is 14 kb). Indeed, mini- or microdystrophins that have ameliorating effects in *mdx* mice have been generated (23, 24). Current efforts are under way to translate this into the clinic. Interestingly, a recent study demonstrated that AAV expressing microdystrophin was well tolerated, and the microdystrophin was strongly expressed upon systemic application in nonhuman primates (25). Based on these experiments, a first Phase I trial on a few DMD patients is currently under way (see **http://www.clinicaltrials.gov;** identifier: NCT00428935).

Pharmacological Approaches that Target the Mutated Gene

A more recent approach to muscular dystrophies is the repair of the endogenous gene by exon-skipping, a strategy that induces the bypass of the exon containing the allelic mutation. The goal of exon-skipping is to exclude mutated exons in the mature mRNA encoding dystrophin (e.g., those exons carrying a nonsense mutation that causes premature stop of translation) by preventing their inclusion during mRNA splicing (**Figure 2*a***). This method should thereby result in a smaller dystrophin protein that is still functional. Exon-skipping uses antisense oligonucleotides that are specifically designed to block the sites recognized by the splicing machinery to process precursor mRNA into the mature mRNA (**Figure 2*a***; 26). Exon-skipping is probably the most promising approach today to substantially alleviate symptoms in DMD, given its solid scientific rationale and the fact that it targets the primary cause of DMD, namely the mutated *dystrophin* gene.

There are currently three distinct approaches that use exon-skipping. Although the principle of antisense-mediated exon-skipping is the same, the three methods differ largely in the chemistry of the oligonucleotides and in the manner by which the antisense sequences are applied to the tissue. In one method, gene therapy using AAV is used to generate a specific U7 small nuclear RNA (U7 snRNA). U7 snRNA is involved in the 3' end processing of the precursor mRNA of histones (27). Proof-of-concept studies in *mdx* mice have provided strong evidence for the potential of this method for the treatment of DMD (28). As packaging capacity of the virus is not an issue with this method, AAV vectors are used. Moreover, if successful, only one-time delivery of the virus



would be needed for a life-long expression of the U7 snRNA. This method is currently undergoing further development using the golden retriever model for DMD (GRMD). Although the method is appealing, the use of AAV also has its limits and its challenges. For example, the virus must be applied systemically and must transduce all skeletal muscles to be effective. The same issues also apply to gene therapeutic approaches using AAV (see review in Reference 21) and, thus, it remains to be seen how quickly this approach can advance to clinical trials.

The other method of exon-skipping involves the direct application of antisense oligonucleotides (AONs) to the tissue. The technique of AON treatment was developed more than two decades ago. After many initial failures in clinical trials, the method has now reached a stage where it can be applied in a clinical setting (for review, see Reference 29). For DMD treatment, the use of AONs is much more advanced into clinical development than the above-mentioned virus-based method. Currently, two chemically distinct AONs are being tested that differ mainly in their backbone. The backbone is largely responsible for compound stability and is also most likely to be the source of potential side effects. The currently tested modifications are 2′-O-methyl-phosphorothionate RNA oligonucleotides (2′OMe nucleotides) and morpholino phosphorodiamidate oligonucleotides (PMOs). Although the clinical development of 2′OMes is further along than that of the PMOs, the most compelling preclinical evidence stems from the use of PMOs. For example, PMOs have been shown to work after local injection and systemic delivery in *mdx* mice (30), and systemic delivery has also been used in the treatment of a beagle crossbreed of the GRMD dogs (31). Importantly, proof-of-concept studies in humans have recently been published for both types of oligos (32, 33). In both trials, efficacy of treatment was determined by measuring the levels of dystrophin after one-time injection of a leg (*tibialis anterior*) or a foot muscle (*extensor digitorum brevis*). In both studies, the AONs proved to be safe and were shown to significantly increase the levels of dystrophin, although the design did not allow determination of whether the amount of dystrophin was sufficient to restore muscle function. In this context, it is interesting to note that the development of highly sensitive and quantitative methods allowing detection of dystrophin in small samples will be important for further trials where the AONs will be applied systemically. Recent progress in the field may allow such quantitative measurements in the near future (34, 35).

What are the challenges for antisense-mediated exon-skipping? Issues that are inherent to the method are that even the successful treatment of a DMD patient will probably not result in a cure but in the conversion into a BMD-like phenotype. In addition, the mutation in *dystrophin* must be characterized for each patient, and only a subpopulation of DMD patients can potentially benefit from this method. Current estimates predict that approximately 15% of the DMD patients would benefit from the skipping of exon 51 of *dystrophin*, and this represents the largest fraction (36).

Figure 2

Mechanism of exon-skipping and premature termination codon (PTC) readthrough. (*a*) Example of a mutation in *dystrophin* that can be treated by exon-skipping. (*Top*) In normal individuals, the primary transcript of *dystrophin* still contains introns and exons (*boxes*). Splicing removes all the introns, thus generating the mature transcript that is exported into the cytoplasm where it is translated into the dystrophin protein. Domains of dystrophin are actin-binding domain (*red*), rod-like domain (*blue*), WW domain (*gray*), cysteine-rich domain (*green*), and the β-dystroglycan interaction domain (*orange*). (*Middle*) A mutation in exon 51 (*pink*) that causes a frameshift will result in a shorter dystrophin protein that lacks the essential β-dystroglycan binding site but contains a short, out-of-frame amino acid stretch (*yellow*). (*Bottom*) An antisense oligonucleotide (AON) that hybridizes to the splice junction of exon 51 (*red line*) will induce the skipping of exon 51 and result in a shorter mRNA that gives rise to a shorter dystrophin protein with all the important domains still present. (*b*) A nonsense mutation in exon 51 can generate a PTC that causes the ribosome to fall off the mRNA prematurely (*top*), resulting in a truncated dystrophin (*dark yellow*). In the presence of a compound that suppresses nonsense codons (*pink pentagon*), ribosomes are able to read through the entire mRNA and, thus, produce full-length dystrophin (*dark yellow*).

Skipping exon 51 could be accomplished by one set of AONs, although it is not entirely clear whether the efficacy of skipping would indeed be the same irrespective of the mutation. For those DMD patients who cannot benefit from skipping exon 51, new AONs need to be designed. The second most common mutations that can be treated by one single AON are found in approximately 8% of DMD patients (36). In total, approximately half of the Duchenne patients could benefit from exon-skipping, but it is important to note that the fraction of patients that can potentially benefit from a particular AON may become very small. The fact that not all DMD patients can be treated with this approach reinforces the need for continued work on alternative approaches (see below).

The biggest challenges faced by the antisense treatment are the need to reach all muscles and the requirement that the AONs penetrate into the myonuclei in sufficient quantity to allow re-expression of dystrophin. Current estimates indicate that 30% of the dystrophin level found in controls is sufficient to avoid a muscle dystrophy (37). Thus, systemic application of the AONs is mandatory. In the ongoing Phase II clinical trial, systemic delivery of a 2′OMe oligonucleotide via weekly subcutaneous injection is being tested using a dose-escalation protocol (see **http://www.clinicaltrials.gov**). As in the Phase I trial, the primary readout will be the levels of dystrophin achieved—but patients will also be tested for functional improvements.

Another challenging factor is that AONs would need to be given to children over many years. Currently implemented treatments with AONs are only for shorter periods and, thus, it is not clear whether long-term treatment may cause some severe side effects. Another important issue is the fact that newly designed AONs of the same chemical class are considered new chemical entities, which need to go through the entire regulatory process for approval. Such a lengthy and expensive process carries the risk that the hurdles to develop AONs for small populations and/or indications are too high for a commercial entity. A possible solution could be fast-track approval of all chemical classes of AONs. The severity of DMD and the relatively small number of patients may require that federal agencies use nonstandard policies to allow access to clinical trials.

In summary, we believe the potential of these new approaches to be high because they are based on a solid scientific rationale and a well-defined mechanism. Nevertheless, the challenges faced are still substantial, and it is still too early to tell whether exon-skipping will become a viable treatment option for even a subpopulation of DMD patients.

Nonsense Suppression

Another potential therapy that also targets the mutated *dystrophin* gene and only targets a rather small subpopulation of DMD patients is the so-called targeted nonsense suppression or premature termination codon (PTC) readthrough. This approach can be used to suppress any nonsense mutation that causes premature termination of translation. The molecular mechanism of nonsense codon suppression is not known in detail (**Figure 2*b***). One obvious concern regarding this method is that the suppression technique must recognize premature stop codons on the one hand, but must not lead to readthrough of true stop codons on the other hand. Thus, the mechanisms must be able to distinguish between those stop codons that are the result of nonsense mutations and the naturally occurring stop codons. Although it is still rather controversial whether such molecular discrimination can be achieved, there is some evidence that the context and mRNA structure near premature stop codons are different from those at a natural stop codon. One such mechanism that might contribute to this discrimination is nonsense-mediated mRNA decay (reviewed in Reference 38).

Drugs that mediate nonsense suppression can potentially be applied to several diseases in specific subpopulations. It also represents, like exon-skipping, an example of personalized medicine.

In the case of DMD, nonsense suppression is potentially applicable to 10–15% of all the patients, and there is evidence that PTC readthrough may be beneficial. For example, the antibiotic gentamicin, which belongs to the class of aminoglycosides and is known to read through PTCs, has been shown to have some efficacy in *mdx* mice that carry a nonsense mutation in exon 23 of *dystrophin* and in a small clinical trial in patients suffering from cystic fibrosis (39, 40). However, in both cases, negative results in similar studies have been reported (41, 42), indicating that the effect of gentamicin on PTC readthrough may not be strong enough to see a beneficial result in a large cohort. Moreover, gentamicin can cause severe kidney damage and ototoxicity.

A high throughput screening approach based on a luciferase reporter assay has yielded several candidate compounds that promote PTC readthrough with high potency (43). Among those, PTC124, an orally available and highly potent compound, was selected for further in vivo studies. Although the usefulness of the initial screening assay to identify nonsense suppressors has recently been questioned (44), PTC124 was shown to ameliorate disease in *mdx* mice (43) and was shown to be efficacious in a Phase II clinical trial in cystic fibrosis (45). Despite this initial success, the most advanced, Phase IIb clinical trial of 174 DMD patients, who were treated for 48 weeks in a double-blind, placebo-controlled design with a high and a low dose of PTC124 (now renamed to Ataluren), has been suspended. Based on recent press releases of PTC Therapeutics, no efficacy in a 6-min walk test was observed in the high-dose group, whereas some improvement was seen in the low-dose group. Although in-depth analysis is still ongoing, these results are disappointing and bring into question whether nonsense suppression remains a promising treatment option, though the current results might be a function of the particular efficacy of the molecule being treated, rather than with the reasoning underlying the approach. It will be interesting to see the results of the ongoing Phase III trial on cystic fibrosis, where long-term improvement of relevant disease parameters will be measured (see **http://www.clinicaltrials.gov** for details). The latest clinical trial with DMD patients was financially supported by patient organizations, one such being the Parent Project Muscular Dystrophy organization, and updated information can also be found at their Web site (e.g., **http://www.parentprojectmd.org**).

MOLECULAR MECHANISMS OF MUSCLE ATROPHY AND HYPERTROPHY

Muscle atrophy and wasting are common features of most muscular dystrophies but muscle loss is a more common phenomenon that is found in many other conditions. For example, cachexia, as seen in settings of cancer, burns, COPD, and renal disease, represents the severe loss of body mass—with a particular emphasis on the loss of muscle. Although the etiology of cachexia is very different from muscular dystrophies, there is evidence of a molecular relationship between the diseases. For example, the dystrophin glycoprotein complex (**Figure 1**) has recently been implicated in the genesis of cancer cachexia (46), thereby providing a molecular link to muscular dystrophies (see also Reference 47).

The IGF-1/PI3K/Akt Pathway Regulates Muscle Mass

In recent years there has been strong evidence for a central role of a signaling pathway activated by insulin-like growth factor-1 (IGF-1) in the control of muscle mass (47, 48). IGF-1 can increase muscle mass in part by stimulating the pathway containing phosphatidylinositol-3 kinase (PI3K) and Akt, also known as protein kinase B (PKB), which in turn results in the activation of downstream targets that induce protein synthesis (49, 50). Weight-bearing or anabolic exercise leads to activation of the PI3K/Akt pathway by directly inducing muscle expression of IGF-1 (51, 52),

which is sufficient to induce hypertrophy of skeletal muscle (53), as was demonstrated in transgenic mice in which IGF-1 is overexpressed in skeletal muscle (54, 55). Activation of Akt is sufficient to induce hypertrophy in vivo, as was shown by the production of transgenic mice in which a mutant, constitutively active form of Akt is conditionally expressed in adult skeletal muscle (56–58). In that setting, acute activation of Akt, for 2–3 weeks, is sufficient to induce a doubling in the size of skeletal muscle; this increase occurs via an increase in the average cross-sectional area of individual muscle fibers (56). Conversely, in settings of skeletal muscle atrophy, Akt activation is downregulated (59). Disruption of the dystrophin glycoprotein complex, which is seen in mouse models of cancer cachexia, results in an upregulation in the E3 ubiquitin ligases that mediate muscle atrophy (46 and see below), which are normally regulated by Akt; this is perhaps due to a dissociation of the cytoplasmic signaling complexes from the extracellular matrix, where muscle fibers normally receive growth factor stimulation (47).

A Hypertrophy Mediator Downstream of PI3K and Akt: mTORC1

Genetic experiments using *Drosophila* originally helped to define a pathway, which included PI3K and Akt, that can control cell size (**Figure 3**). This is the pathway that is recruited by IGF-1 in mammals. In mammals, IGF-1 induces phosphorylation of the IGF-1 receptor, a tyrosine kinase that activates, in sequence, the insulin receptor substrate (IRS; 60), PI3K (61), and Akt. Activation of Akt inhibits the protein complex of tuberous sclerosis complex 1 (TSC1 or hamartin) and tuberous sclerosis complex 2 (TSC2 or tuberin) and thereby removes inhibition of Ras homolog enriched in brain (Rheb), which, in turn, activates the mammalian target of rapamycin (mTOR). mTOR can assemble into two distinct multiprotein complexes, named mTOR complex 1 (mTORC1) and mTORC2 (62, 63). It now is clear that the main pathway acting downstream of Akt in skeletal muscle is mTORC1 (64, 65) and not mTORC2. Activation of mTORC1 then causes phosphorylation of p70 S6 kinase 1 (S6K; 66) and 4E-BP (also known as PHAS-1), which is a negative regulator of the protein initiation factor eIF4E (67, 68). Thus, activation of mTORC1 results in increased protein translation. Although IGF-1 activates mTOR and S6K downstream of PI3K/Akt activation, amino acids can activate mTOR directly, causing a subsequent stimulation of S6K activity (69, 70). Thus, mTOR appears to have an important and central function in integrating a variety of growth signals, from simple nutritional stimulation to activation by protein growth factors, resulting in protein synthesis.

The drug rapamycin binds to FKBP12, which, in turn, binds to mTOR and inhibits its function (71–73). In vitro, when applied to myotube cultures, rapamycin blocks activation of S6K downstream of either activated Akt or IGF-1 stimulation (50, 73, 74) (**Figure 3**). However, rapamycin does not completely block IGF1-mediated hypertrophy in vitro, which indicates that other pathways downstream of Akt but independent of mTOR play a role in some settings of hypertrophy. mTORC1 includes a protein named raptor—this complex can be inhibited by rapamycin (75–77). In the rapamycin-insensitive complex mTORC2, the protein rictor is necessary for its function (62, 63). This complex is able to phosphorylate several AGC [cAMP-dependent protein kinase (PKA), protein kinase G, protein kinase C (PKC)] kinases (reviewed in Reference 78). Among those, Akt becomes activated by mTORC2 by a positive feedback mechanism (79).

The mTORC1 target 4E-BP can directly bind raptor (75, 76). Mutations in 4E-BP that inhibit interaction with raptor also inhibit mTOR-mediated phosphorylation of 4E-BP (80). Finally, overexpression of raptor can enhance the phosphorylation of 4E-BP by mTOR in vitro (80, 81). mTOR binds 4E-BP by a TOR signaling (TOS) motif; this same motif is found in S6K (81), demonstrating both a mechanism for mTOR's interaction with its downstream signaling components and the possibility that there may be some selective hierarchy in signaling (because

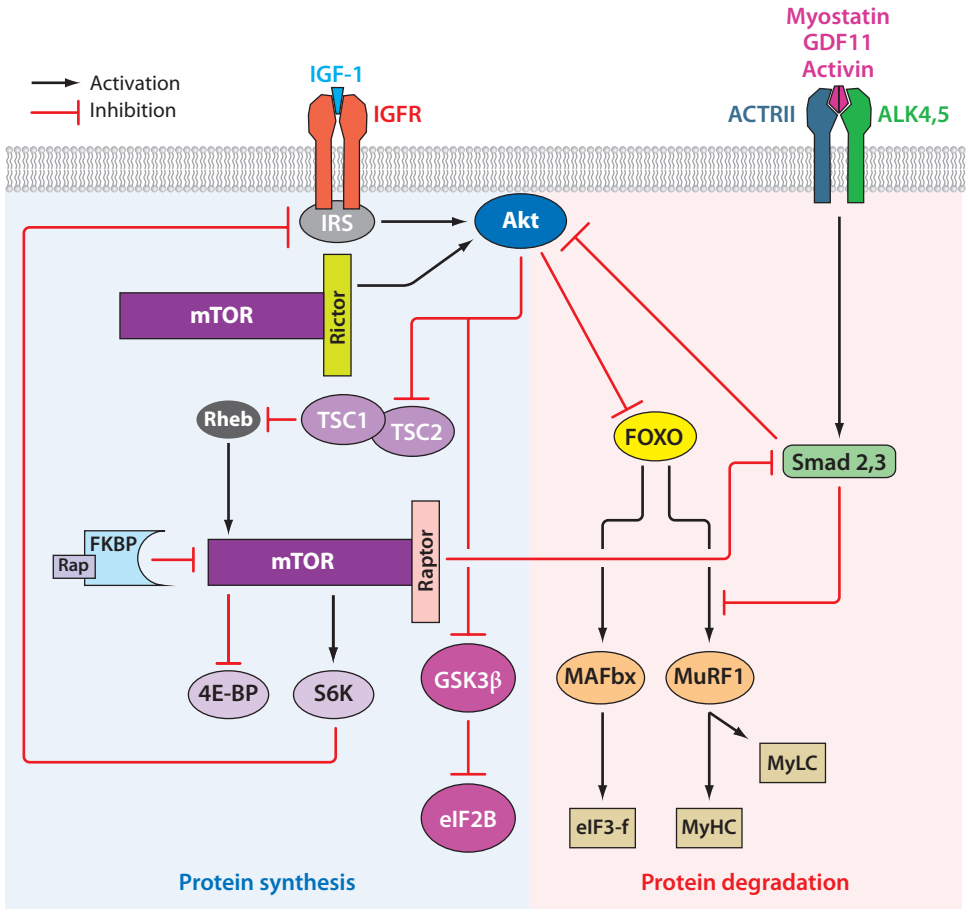


Figure 3

Schematic representation of the signaling pathways involved in the control of muscle atrophy and hypertrophy. Signaling activated by insulin-like growth factor-1 (IGF-1) and that activated by myostatin/TGFβ are the two best-characterized pathways that regulate muscle size. IGF-1 acts via the IGF receptor (IGFR), insulin receptor substrate (IRS), and Akt (also termed PKB). Akt activates mTOR complex 1 (mTORC1) as it removes inhibition of the tuberous sclerosis complex 1 or 2 (TSC1/TSC2) protein complex onto Ras homolog enriched in brain (Rheb). mTORC1 is a multiprotein complex that requires the protein raptor for its function and is acutely inhibited by FKBP/rapamycin. mTORC1 controls protein synthesis by phosphorylating S6 kinase 1 (S6K) and eIF4E-binding protein (4E-BP). The multiprotein complex mTORC2 includes the protein rictor and contributes to the activation of Akt. Downstream targets of Akt include glycogen synthase kinase 3β (GSK3β) and Forkhead box O (FOXO) transcription factors. Inhibition of GSK3β by Akt relieves inhibition onto the initiation factor eIF2B, and thereby increases protein synthesis. Activation of Akt also inhibits FOXO and decreases expression of the E3 ubiquitin ligases Muscle Atrophy F-box (MAFbx) or Atrogin-1 and Muscle Ring Finger1 (MuRF1). Substrates of MAFbx and MuRF1 are the initiation factor eIF3-f and myosin chains, respectively. The myostatin/TGFβ pathway acts via several receptors and results in the activation of Smad 2,3. Activation of Smad proteins inhibits the function of Akt and the expression of MAFbx and MuRF1 by FOXO transcription factors. The function of Smad 2,3 is also inhibited by mTORC1.

the same motif binds mTOR, one might wonder if there is competition for the same mTOR interaction site). In summary, mTORC1 can increase protein synthesis by modulating two distinct pathways, the S6K pathway and the 4E-BP pathway (**Figure 3**).

A Second Hypertrophy Mediator Downstream of PI3K and Akt: GSK3β

Glycogen synthase kinase 3β (GSK3β) is a distinct substrate of Akt that can modulate hypertrophy. Phosphorylation by Akt inhibits GSK3β activity (82). Expression of a dominant-negative kinase inactive form of GSK3β induces significant hypertrophy in myotubes (50), as does inhibition of GSK3 with lithium (83). GSK3β blocks protein translation initiated by the eIF2B protein (84) and blocks differentiation induced by a transcription factor named nuclear factor of activated T cell (NFAT; 50, 85). Inhibition or loss of GSK3β protein activity results in enhanced myotube formation and muscle-specific gene expression during differentiation (85). In addition, GSK3β inhibition restores myogenic differentiation following calcineurin blockade, which further implicates the involvement of the transcription factor NFAT in myoblast differentiation downstream of IGF-1 activation (85). Furthermore, GSK3β-deficient myoblasts demonstrated enhanced nuclear translocation of NFATc3 and elevated NFAT-sensitive promoter transactivation (85). IGF-1-mediated inhibition of GSK3β is, therefore, a distinct mechanism for inducing hypertrophy and promoting myoblast differentiation.

Skeletal Muscle Atrophy Occurs via Induction of Distinct E3 Ubiquitin Ligases, Whose Expression Can Be Inhibited by IGF-1

As mentioned above, skeletal muscle atrophy occurs in a variety of settings. There is a distinct set of genes that are inversely regulated under IGF-1-induced hypertrophy conditions in dexamethasone (a glucocorticoid)-induced atrophy (86); these include the gene *MAFbx* (*Muscle Atrophy F-box*; 87), which is also known as *Atrogin-1* (88). A second gene, *MuRF1* (*Muscle Ring Finger1*) is significantly upregulated under atrophy conditions (87). Both *MuRF1* and *MAFbx/Atrogin* were shown to encode E3 ubiquitin ligases (87). Expression of *MuRF1* and *MAFbx* is stimulated in over a dozen distinct models of skeletal muscle atrophy (87–91). Mice that are null for *MuRF1* (*MuRF1*$^{-/-}$) and mice null for *MAFbx* (*MAFbx*$^{-/-}$) appear phenotypically normal. However, under atrophy conditions, significantly less muscle mass is lost in either *MuRF1*$^{-/-}$ or *MAFbx*$^{-/-}$ animals in comparison with control littermates (87).

MuRF1 encodes a protein that contains three domains: a RING-finger domain (92), which is required for ubiquitin ligase activity (93); a "B-box," whose function is unclear, and a "coiled-coil domain," which may be required for the formation of heterodimers between MuRF1 and a related protein, MuRF2 (94). Proteins that have these three domains have been termed RBCC proteins (for RING, B-BOX, coiled-coil domain; 95) or TRIM proteins (for tripartite motif; 96). MuRF1 has been demonstrated to have ubiquitin ligase activity that depends on the presence of the RING domain for that activity (87). MuRF1 has been shown to bind to the myofibrillar protein titin at the M line (94, 97, 98).

MuRF1 and myosin heavy chain (MYH) physically interact—as demonstrated by immunoprecipitation of epitope-tagged MuRF1 protein, which co-immunoprecipitated MYH protein; this finding led to the discovery that MYH was a substrate of MuRF1 (99). Subsequently, it was shown that several other proteins in the thick filament of muscle were also degraded by MuRF1, including myosin light chain and myosin binding protein C (100). Therefore, MuRF1 induces muscle atrophy in part by directly attacking the thick filament of the sarcomere by mediating the proteolysis of myosin proteins.

MAFbx/Atrogin-1 contains an F-box domain, a characteristic motif seen in a family of E3 ubiquitin ligases named SCFs (for Skp1, Cullin, F-box; 101). F-box containing E3 ligases usually bind a substrate only after that substrate has first been posttranslationally modified, for example, by phosphorylation (101). This suggests the possibility of a signaling pathway in which a potential substrate is first phosphorylated in response to an atrophy-induced stimulus and then degraded via MAFbx.

Substrates have been suggested for MAFbx, including MyoD (102) and calcineurin (103). However, it has not yet been shown whether either protein is ubiquitinated by MAFbx in skeletal muscle or only under atrophy conditions. In cardiac muscle, it was shown that while MAFbx has no effect on Akt activation in response to IGF-1 or insulin challenge in cardiomyocytes, nevertheless MAFbx can repress Akt-dependent hypertrophy by activating the Forkhead transcription factors via a distinct type of ubiquitination—ubiquitination using Lysine 63, which perturbs transcriptional activity (in this case that of the FOXO transcription factors) rather than inducing proteasomal degradation (104); FOXO (Forkhead box O) activation was shown to be required to activate the atrophy transcriptional program (105), as is discussed further in the next section. Because FOXO proteins regulate MAFbx expression in skeletal and cardiac muscle, these findings indicated the presence of a feed-forward mechanism in which MAFbx is activated by, and in turn coactivates, FOXO3a and FOXO1 (104), making it clear why IGF-1's ability to inhibit FOXO via activation of Akt is necessary to inhibit uncontrolled atrophy in skeletal muscle. More recently, MAFbx has been shown to be an E3 ligase for eIF3-f, a protein initiation factor (106). This finding suggests that MAFbx results in muscle atrophy by down-regulating protein synthesis through the proteolysis of a required protein initiation factor.

IGF-1/PI3K/Akt Inhibition of FOXO Transcription Factors Blocks Upregulation of MuRF1 and MAFbx

Studies of differentiated myotube cultures demonstrated that treatment of myotubes with dexamethasone promotes enhanced protein breakdown and increased expression of genes broadly involved in the ubiquitin-proteasome proteolytic pathway (107–109). In vitro treatment of myotubes with dexamethasone induces atrophy, accompanied by the specific increased expression of *MAFbx* and *MuRF1* (105, 110). The upregulation of *MAFbx* and *MuRF1* was antagonized by simultaneous treatment with IGF-1 (105, 110, 111), acting through the PI3K/Akt pathway (105, 110); this finding demonstrated a novel role for Akt—in addition to stimulating skeletal muscle hypertrophy, Akt stimulation could dominantly inhibit the induction of atrophy signaling (**Figure 3**). Similarly, in a separate model of atrophy, diabetes, *MuRF1* and *MAFbx* were activated and here too IGF-1 blocked the transcriptional upregulation (112). Genetic activation of Akt was shown to be sufficient to block the atrophy-associated increases in *MAFbx* and *MuRF1* transcription (110). The mechanism by which Akt inhibited *MAFbx* and *MuRF1* upregulation was demonstrated to involve the FOXO family of transcription factors (105, 110, 112). In myotubes, FOXO transcription factors are excluded from the nucleus when phosphorylated by Akt and translocate to the nucleus upon dephosphorylation. The translocation and activity of FOXO transcription factors are required for upregulation of *MuRF1* and *MAFbx*—in the case of FOXO3, activation was demonstrated to be sufficient to induce atrophy (105), a finding that was subsequently supported by the transgenic expression of FOXO1, which also resulted in an atrophic phenotype (113).

IGF-1 Regulation of Myostatin

In addition to IGF-1, other secreted proteins have been demonstrated to perturb skeletal muscle size. Myostatin, also termed GDF-8, is a TGFβ family member that is a negative regulator of

muscle mass (112). Myostatin's effect was demonstrated in studies with mice that were made null for the myostatin gene (114) and also by correlating increases in muscle mass that were observed in strains of cattle with a loss of myostatin (115–117); the loss of myostatin resulted in a more than doubling in muscle mass. It has been suggested that other TGFβ superfamily molecules, distinct from myostatin, play a role in modulating skeletal muscle size, because myostatin$^{-/-}$ mice that are mated with mice that are transgenic for follistatin (TG^{follistatin}), which is capable of inhibiting not only myostatin but also its close relative GDF-11 and other TGFβ molecules such as the activins, resulted in an even greater increase in muscle size (118).

In vitro studies with myostatin have been performed on rodent cells. In these studies, it has been shown that myostatin can block the differentiation of myoblasts into myotubes (119–122). Experiments both in vitro and in vivo have demonstrated that myostatin signals by first binding to the type II activin receptor IIb, which then allows for interaction with type I receptors ALK4 or ALK5 (**Figure 3**; 123). The binding of myostatin to these receptor complexes results in the phosphorylation and activation of the transcription factors Smad2 and Smad3, which translocate to the nucleus upon phosphorylation (124). In a study of myostatin and other TGFβ molecules on human skeletal myoblasts (HuSkMC) and myotubes, HuSkMCs respond to myostatin at physiologic concentrations (0.1 to 300 ng ml^{-1}), resulting in a decrease in fusion index, myotube diameter, creatine kinase activity, and expression of MyoD and myogenin (125). It was previously demonstrated that follistatin, a more general inhibitor of TGFβ, could induce an additive increase in muscle mass when combined with myostatin (118). A range of other TGFβ molecules are able to block muscle differentiation, including the more distantly related activins and BMP-2 (125). Myostatin inhibits activation of Akt in both myoblasts and myotubes (125). It was recently reported that muscle-specific ablation of mTORC1 (by ablating *raptor*) results in a dystrophic phenotype (64). Inhibition of raptor, and thus mTORC1, contributes to myostatin's inhibitory effects, resulting in an increase in myostatin-induced Smad phosphorylation and establishing a feed-forward mechanism: Myostatin activates Smad2, which inhibits Akt and its downstream target mTORC1, which in turn potentiates myostatin's activation of Smad2. These findings are outlined in **Figure 3**.

Addition of IGF-1 dominantly blocks the effects of myostatin, when applied to either myoblasts or myotubes (125). The precise intersection between the two pathways may be multifold, but it is clear that Akt is a particular nexus and that IGF-1 can rescue the activation of Akt that is blunted by myostatin.

The demonstration that IGF-1 can dominantly overcome myostatin inhibition adds to the rationale for IGF1-based treatment regimens in clinical settings, where myostatin is active. Thus, the detailed knowledge of the signaling pathways involved in the control of muscle size has set the stage for the development of pharmacological approaches to interfere with the pathways.

PGC1α Induction Induces Mitochondriogenesis and Fiber-Type Switching

In addition to perturbations in protein synthesis and degradation, another major system that is affected during long-term atrophy is a decrease in mitochondria (126). The transcriptional coactivator Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α) has been shown to be sufficient to positively modulate mitochondriogenesis (127). There are two reports that this factor can also block skeletal muscle atrophy, perhaps by negative regulation of FOXO signaling (128, 129). Interestingly, one of the known signaling pathways that increases mitochondriogenesis via PGC1α is the activation of AMPK, which at the same time actually decreases protein synthesis by blocking mTOR (130). Therefore, it is of interest to see if it is possible to positively modulate the PGC1α pathway without blocking protein synthesis, because there is a net loss of protein in atrophy, and therefore an additional inhibition in synthesis pathways may not be desirable.

Conclusion

In conclusion, considerable progress has been made in the understanding of the signaling pathways that mediate skeletal muscle hypertrophy and atrophy. Whereas it was appreciated many years ago that hypertrophy comes about via an increase in the rate of protein synthesis and atrophy through an increase in protein degradation, only now can specific signaling pathways be drawn, because the particular molecular mediators of hypertrophy and atrophy in skeletal muscle have only recently been determined. Furthermore, only through recent studies is it now understood that certain hypertrophy pathways have been shown to be dominant over the induction of atrophy mediators. These findings give hope that novel drug targets may be found to block the skeletal muscle atrophy seen in a variety of clinical conditions, from the cachexia of AIDS, sepsis, and cancer to the gradual loss of muscle mass observed during normal aging.

ARE THE SIGNALING PATHWAYS INVOLVED IN ATROPHY ALSO SUITABLE TARGETS FOR THE TREATMENT OF MUSCULAR DYSTROPHIES?

As has been highlighted above, the most promising strategies in the treatment of muscular dystrophies, in particular DMD, target the disease-causing *dystrophin*. Muscle wasting is also a characteristic feature of muscle dystrophies, and it is thus natural to think that treatments preventing muscle atrophy may also ameliorate muscle dystrophies. Although this idea is appealing, the initial, causative molecular mechanisms that are perturbed in muscle dystrophies are fundamentally different than those acting in diseases that cause muscle atrophy, even though there is clearly downstream overlap between the conditions. There are many examples that underscore a fundamental distinction, however. For example, glucocorticoids, which are the only medications currently available that slow the decline in muscle strength and function in DMD, cause muscle atrophy in patients who have an unmutated dystrophin allele (131). In addition, and most importantly, the primary cause of the most common muscle dystrophies is a deficit in sarcolemmal stability that triggers the degeneration of muscle fibers, and atrophy and muscle loss are only two of the downstream consequences. After degeneration of muscle fibers, regenerative processes are important in rebuilding muscle. In many muscle dystrophies muscle regeneration is impaired, indicating that agents that improve the regenerative capacity of muscle are also likely to ameliorate the disease phenotype of muscle dystrophies. These considerations indicate that antiatrophy treatments should be considered as second-line treatment options that can provide some benefit in muscle dystrophies but are unlikely to ameliorate disease progression profoundly. Furthermore, some prohypertrophy strategies might actually be detrimental to the dystrophic patient, because anabolic exercise increases myofiber breakdown in such patients (132, 133); in contrast, anabolic exercise has been demonstrated to maintain strength in sarcopenic patients (134, 135).

This caution regarding antiatrophy treatments should remain even given the finding that a loss of dystrophin can activate proatrophic signaling pathways; this does not necessarily mean that blocking these pathways will ultimately be beneficial to patients with mutations in genes encoding components of the dystrophin glycoprotein complex. However, there are some promising data that at least justify the careful exploration of cross-application of these pathways. As described in detail above, there are two main pathways involved in the control of muscle mass, the myostatin pathway and the IGF-1 pathway. These two pathways overlap with the ability of IGF-1 to suppress myostatin-induced loss of muscle (125, 136). The usefulness of targeting both pathways for the treatment of muscle dystrophies has been tested in preclinical animal studies with rather promising results. Overexpression of IGF-1 in skeletal muscle in *mdx* mice (137), or

administration of IGF-1 by a mini-pump (138), ameliorated several of the disease parameters. These include an increase in the cross-sectional area of myofibers and some protection against contraction damage. Interestingly, some investigators also reported an increase in the number of oxidative muscle fibers and a reduction of fast-twitch, type II fibers (e.g., 139). Oxidative, slow-twitch fibers (type I) have been reported to be less prone to damage in DMD patients (140). In addition, oxidative fibers express higher levels of utrophin (141), an autosomally-encoded homolog of dystrophin that can compensate for the loss of dystrophin (142). It is also interesting to note that activation of Akt (which is downstream of IGF-1; see **Figure 3**) has an ameliorating effect in *mdx* mice (143, 144). However, this may occur by a distinct, unexpected mechanism, as activation of Akt was also shown to increase levels of utrophin (143, 144). Thus, the improvement observed may rely on this increase rather than the induction of muscle hypertrophy. Interestingly, muscle-specific deletion of *mTOR* or its interacting protein raptor, which are downstream components to Akt, causes a dystrophic phenotype (64, 65). In the case of the mTOR knockout, expression levels for dystrophin are lower.

In summary, activation of the lGF-1 pathway not only leads to a compensatory hypertrophy but also affects pathways whose function is lost in muscle dystrophies. Although the detailed mechanisms by which IGF-1 affects these pathways have not been elucidated, the possibility that IGF-1 affects expression of structural genes that are causative to the disease makes this a more attractive approach to treatment than if IGF-1 simply induces muscle hypertrophy without improving the underlying mechanisms for a dystrophic phenotype. Despite the on-target findings, there are still many challenges to be faced. For example, IGF-1 is expressed in several isoforms, and it is not clear whether all isoforms are equally potent in skeletal muscle (reviewed in Reference 145). In addition, IGF-1 released into the blood is bound to several binding proteins that are important for its stabilization and function. IGF-1 is also involved in the control of cell and organ size systemically, which makes the use of IGF-1 for life-long treatment problematic in patients who already have normal levels of IGF-1. Finally, supraphysiologic levels of IGF-1 can cause hypoglycemia, because high concentrations of IGF-1 stimulate the insulin receptor (146).

Current progress in testing the role of myostatin in muscle dystrophies suggests that blockade of myostatin signaling may show some benefit, although rather transiently. Blockade of myostatin by antibody application or overexpression of a dominant-negative form of myostatin has been shown to improve some of the hallmarks of the disease in *mdx* mice (147–149). As *mdx* mice are not as severely impacted as are DMD patients (for example, their life span is not shortened), it is difficult to predict whether or not the hypertrophic response of muscle to myostatin blockage might be counterproductive in human patients. For example, it is noteworthy that elimination of myostatin signaling in laminin-α2 deficient mice, the mouse model for the congenital muscle dystrophy MDC1A, worsens rather than ameliorates disease progression (150). Cross-breeding of whippet dogs that are deficient for myostatin (151) and GRMD results in a worsening of the phenotype (J.N. Kornegay, personal communication). Moreover, in some cases, the improvement was rather transient (149). All these data suggest that myostatin inhibition might be of limited value in DMD. In addition, a recent, double-blind, placebo-controlled Phase I/II trial using antibodies to inhibit myostatin was not efficacious (152). However, the trial was not powered to see efficacy and, thus, the question remains as to whether inhibition of myostatin could be beneficial for muscle dystrophies.

DISCLOSURE STATEMENT

D.J.G. is a stockholder and employee of Novartis Institutes for BioMedical Research; M.A.R. is cofounder, advisor, and shareholder of Santhera Pharmaceuticals Ltd. In the view of the authors, these affiliations did not affect the objectivity of this review.

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