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Outside in: the matrix as a modifier of muscular dystrophy

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Abstract

Muscular dystrophies are genetic conditions leading to muscle degeneration and often, impaired regeneration. Duchenne Muscular Dystrophy is a prototypical form of muscular dystrophy, and like other forms of genetically inherited muscle diseases, pathological progression is variable. Variability in muscular dystrophy can arise from differences in the manner in which the primary mutation impacts the affected protein's function; however, clinical heterogeneity also derives from secondary mutations in other genes that can enhance or reduce pathogenic features of disease. These genes, called genetic modifiers, regulate the pathophysiological context of dystrophic degeneration and regeneration. Understanding the mechanistic links between genetic modifiers and dystrophic progression sheds light on pathologic remodeling, and provides novel avenues to therapeutically intervene to reduce muscle degeneration. Based on targeted genetic approaches and unbiased genomewide screens, several modifiers have been identified for muscular dystrophy, including extracellular agonists of signaling cascades. This review will focus on identification and possible mechanisms of recently identified modifiers for muscular dystrophy, including osteopontin, latent TGFβ binding protein 4 (LTBP4) and Jagged1. Moreover, we will review the investigational approaches that aim to target modifier pathways and thereby counteract dystrophic muscle wasting.

Keywords

Duchenne muscular dystrophy; genetic modifiers; SPP1; osteopontin; LTBP4; Jagged1; TGFβ; Notch; myostatin; novel drugs; investigational medicinal products; monoclonal antibodies

Introduction: muscular dystrophy and genetic modifiers

Muscular dystrophies are inherited conditions leading to progressive wasting of striated muscle. The most common form in children is Duchenne muscular dystrophy (DMD). DMD

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Conflicts of Interest: EMM has provided consulting services for Novartis, Invitae, Mitobridge, Summitplc, AstraZeneca, and Pfizer and served as a data safety monitor for Eli Lilly and Fibrogen, and has patent application 13/957,100 "Mitigating tissue damage and fibrosis via latent TGFβ protein (LTBP4).

occurs in approximately 1:3500 births; in populations with access to carrier screening and prenatal diagnosis, the incidence is 1:5000 – 1:10000 [1]. DMD is caused by mutations that disrupt the *DMD* gene, which encodes for dystrophin. In myofibers, dystrophin links the actin cytoskeleton to the muscle membrane, the sarcolemma, which helps to maintain a complex of proteins called the dystrophin glycoprotein complex (DGC). The DGC is linked to laminin in the extracellular side of the sarcolemma. Disrupting mutations in dystrophin result in loss of membrane integrity and continuous injury and necrosis of myofibers, which are progressively substituted by fibrofatty tissue. Detrimental remodeling impairs muscle functionality and eventually leads to cardiac and respiratory insufficiency [2]. Heterogeneity in *DMD* mutations is mirrored by variability in severity and characteristics of disease progression. The clinical phenotype can range from severe, with loss of ambulation in early childhood, to mild, as in the case of Becker muscular dystrophy, where *DMD* mutations result in a hypofunctional, but not completely dysfunctional, or absent protein [3, 4]. However, phenotypic variability may occur in patients with the same primary *DMD* mutation, in part explained by partial transcripts produced even in the presence of deletions or frame-shift mutations. Exceptions to the *DMD* reading frame rule are often explained by mutations that may disrupt exon splicing or generate alternative start codons [5]. In addition, a wide-range of clinical manifestations has been reported even in those patients completely lacking dystrophin, suggesting that genetic modifiers can impart an additive effect on dystrophic disease severity [6, 7].

The existence of modifiers of dystrophinopathy has been substantiated by studies in murine models of DMD. The most widely studied genetic model of DMD is the *mdx* mouse, originally identified by means of elevated creatine kinase levels in the circulation [8]. *Mdx* mice bear a premature stop codon in exon 23 of the X-linked dystrophin gene [9]. In mice, phenotypic variability of the same *mdx* mutation ranges from very severe in the DBA/2J strain [10], to intermediate in the C57/BL10 strain, and to very mild in the 129T2/SvEmsJ genetic background [11]. Thus, DMD progression is modified by secondary mutations and polymorphisms that account for inter-individual variability in patients and differences among strains in laboratory mice. The genes affected by secondary variations are called genetic modifiers, as they significantly modify the pathophysiological context of muscle remodeling, and hence the clinical severity of the primary mutation. Identification of genetic modifiers is useful to predict prognosis and unveil pathways that can be therapeutically targeted [12]. Genetic modifiers can be identified with either targeted, or unbiased approaches. Targeted approaches generally assess the effects of genetic manipulation of candidate genes in muscle homeostasis. For example, downregulation of the transforming growth factor β (TGFβ) pathway was shown to mitigate features of muscular dystrophy in mice using a transgene to express a dominant negative TGFβ receptor [13]. Similarly, fibrosis was reduced in the *mdx* mouse by ablating *Spp1*, which encodes osteopontin [14]. Candidate gene approaches have been very useful to identify pathways that alter disease outcomes.

Conversely, genomewide approaches are fundamental to discover unknown candidates since these approaches are largely unbiased. Unbiased studies rely on qualitative or quantitative discrimination of pathological heterogeneity. An example of unbiased approach based on qualitative discrimination has been recently conducted on dystrophic dogs. Within a colony

of dogs bearing the same spontaneous mutation in dystrophin, two exceptional cases stood out because they showed mild dystrophic progression and had a normal lifespan. These two “escaper” animals were then compared to control diseased animals by overlaying whole-genome sequencing data with differences in muscle transcriptional profiles [15]. This study identified *Jagged1* as beneficial modifier of dystrophic pathology.

Another example of an unbiased approach to identifying modifiers, based on quantitative phenotyping, was conducted on a large cohort of dystrophic mice, which shared the same primary mutation on a mixed DBA/2J-129T2/SvEmsJ background. Mice used for this approach were deleted for the *Sgcg* gene encoding the dystrophin-associated protein γ-sarcoglycan. However, similar pathological remodeling downstream of the defective DGC renders this murine model relevant to DMD modifiers [16]. The pathological phenotype of these mice was quantified according to muscle injury and fibrosis parameters, while their genome was analyzed by means of microarray tiling. Overlay of these two datasets led to correlation of specific genomic loci with significant changes in pathophysiologic traits of muscular dystrophy (quantitative trait loci analysis) [17, 18]. This study identified, among others, latent TGFβ binding protein 4 (*LTBP4*) as genetic modifier of muscular dystrophy. Osteopontin, Jagged1, and LTBP4 act as extracellular mediators of signaling cascades, and in this review, we will detail their action on muscular dystrophy from the outside in.

Osteopontin: a multi-faceted modifier

The *Spp1* gene encodes osteopontin (also known as secreted phosphoprotein 1), a secreted glycoprotein that signals through integrin and CD44 receptors. In dystrophic human and mouse muscle, osteopontin mRNA is highly upregulated [14, 19–22]. Comparative expression profiling of skeletal muscle from different dystrophic mouse models showed that *Spp1* is upregulated in both mildly and severely affected dystrophic murine models, as compared to wildtype controls [23]. Intriguingly, these results align with another study where *SPP1* was found as the most upregulated transcript, when comparing the skeletal muscle profiles of dystrophic versus wildtype Golden Retriever dogs at 6 months of age [24]. At the protein level, osteopontin is elevated more than 6 fold in DMD, and between the milder BMD and severe DMD, there is a 2.6 fold difference in protein expression as determined by immunoblotting [25]. A comparison of SPP1 mRNA expression in DMD patient muscle also demonstrated a 2.7 fold increase in samples from diagnostic biopsies of individuals who had a particularly severe clinical course, as compared to biopsies from individuals with a relatively milder course [26].

A polymorphism in the human *SPP1* genomic locus, rs28357094, has been shown to correlate with outcomes in DMD. The SNP rs28357094T>G significantly correlated with a more rapid progression of disease, earlier loss of ambulation, and reduction of grip strength in a cohort of DMD patients [26]. This study overlaid mRNA profiling of severe versus mild DMD patients with genome-wide association studies (GWAS) on healthy volunteers [26]. In a subsequent longitudinal study on ethnically restricted DMD patients from Italy, ambulatory DMD patients with the T allele (*T/T*) were compared to those bearing the G allele (*G/T* and *G/G*). In this study, the G allele significantly correlated with faster deterioration of muscle performance using both the North Star ambulatory assessment and 6-

minute walk test. This correlation was considered suggestive of a dominant model of action for the G allele [27]. Furthermore, the *SPP1* SNP associated with response to glucocorticoid steroids in DMD patients. In a multiethnic cohort, the G allele associated with a 1.2 year earlier median loss of ambulation, and this difference significantly increased to 1.9 years when considering only steroid-treated patients. The same effect size, a 1.9-year difference in loss of ambulation between T/T and *G/T-G/G* genotypes, was confirmed in a sub-cohort including only patients of European or European-American ancestry [28]. However, a multi-center analysis of DMD cohorts across Europe, in which there was no stratification by ethnicity, did not confirm a significant association between the rs28357094T>G SNP and age of ambulation loss. The study relied on a multivariate analysis, taking into account haplotype, steroid regimen and cohort as covariates [29].

In line with the notion that osteopontin promotes dystrophic remodeling and fibrosis, genetic ablation of osteopontin in *mdx* mice resulted in dramatic reduction of fibrosis and concomitant improvement of strength and pathophysiology of dystrophic muscle [14]. Macrophages have emerged as mediators of some of the effects exerted by osteopontin, or its loss, in dystrophic muscles. *Spp1* ablation skewed muscle macrophages from a pro-inflammatory to a pro-regenerative profile, promoting upregulation of insulin-like factor 1 (IGF1), leukemia inhibitory factor (LIF) and urokinase-type plasminogen activator (uPA) [30].

In addition to the role in inflammation, excess soluble osteopontin was found to increase proliferation but decrease fusion and migration of myoblasts [31], consistent with a direct effect on myoblasts to inhibit features needed for efficient regeneration. However, the role of osteopontin in muscle regeneration *in vivo*, and specifically after recovery from injury is more complex. In a study assessing degeneration/regeneration using whole muscle autografting as a model of acute injury, *Spp1*-null grafts had a delay in inflammatory infiltration and regeneration, as compared to wildtype control grafts [32]. These data are distinct from those in [14], and differences between these results may relate to the chronic injury that typifies DMD and/or cell type-specific effects that cannot be assessed by a constitutively deleted allele of *Spp1* in mice.

Data from DMD patients, *mdx* mice and dystrophic dog models suggests that higher levels of osteopontin correlate with promotion of DMD disease [14, 23, 24]. Curiously, the SNP associated with enhanced disease in DMD patients, rs28357094T>G in the human *SPP1* promoter, correlated with weaker promoter activity *in vitro* using luciferase reporter assays [33]. Thus, the association of the G allele (hence, putatively lower *SPP1* expression) with increased disease severity is in apparent contrast with not only the reports of *SPP1* upregulation in muscle of murine models and patients of DMD, as compared to healthy controls [23, 24], but also the traditional view of osteopontin as a pro-inflammatory cytokine [34]. It must be noted that a direct assessment of *SPP1* muscle expression comparing *T/T* versus *T/G* or *G/G* patients did not reveal quantitative changes in *SPP1* mRNA levels in some studies [35],

One reason for this apparent lack of parity may relate to the cell types utilized to interrogate the effect of *SPP1* SNPs through reporter assays. It is critical to discriminate between the

cellular source and the cellular target of osteopontin signaling. Therefore, the use of surrogate cell types for some of the *SPP1* gene expression studies may not adequately mirror gene expression in the context of diseased human tissue, especially one as complex as the DMD striated muscle. In addition, steroid hormones may play a role in *SPP1* regulation. The rs28357094T haplotype reduces the responsiveness of *SPP1* promoter to estrogen-driven transcriptional activation [36]. Moreover, a study performed in malignant astrocytoma cell lines demonstrated binding of the proximal promoter element (surrounding the rs28357094 site) by the glucocorticoid receptor [37]. A potential role of glucocorticoid steroids in the effect size of the rs28357094 SNP might explain disparate results on this modifier; the SNP effect appears to be greater in steroid-treated patients [28] and less detectable in DMD populations where steroid dosing was low [29]. To reconcile these results likely requires examination of gene expression in more relevant cell models in order to more understand the impact of steroid hormones and glucocorticoid regimens on *SPP1* regulation in DMD males.

Another emerging hypothesis on the role of osteopontin as a genetic modifier of muscular dystrophy is the still poorly investigated link between osteopontin and TGFβ signaling. *Spp1* deletion results in decreased levels of intra-muscular TGFβ in *mdx* mice [14]. Accordingly, osteopontin drives *TGFβ1* upregulation, although this was documented in non-muscle mesenchymal cells [38]. Conversely, the *Spp1* promoter is responsive to TGFβ signaling [39], and TGFβ1 is able to increase *Spp1* levels [40]. Moreover, although the mechanisms are still unknown, a polymorphism in the gene encoding for the TGFβ receptor 2 (*TGFBR2*) appeared as a strong predictor of *SPP1* mRNA levels in DMD muscle biopsies [35]. Thus, osteopontin is a multi-faceted modifier of muscular dystrophy via regulation of macrophage polarization and regenerative potential in muscle (Figure 1). Scattered evidence points at direct crosstalk between osteopontin and TGFβ pathways; however, such regulatory circuitry in dystrophic myofibers still awaits a more comprehensive evaluation.

SPP1 is not the only genetic modifier impacting immune cell modulation. Recently, a hypothesis-driven exome screening on DMD patients of European or European-American ancestry identified the rs1883832C>T as modifier of age of loss of ambulation, with the C haplotype associating with earlier age [41]. This SNP falls in the 5′-UTR of *CD40*, which modulates T cell activation and can be found also on the surface of myofibers. Intriguingly, the same group reported a similar effect of the minor SNP haplotype (T) on loss of ambulation in other three independent cohorts of DMD patients [41]. Although the molecular mechanisms and the cell context of CD40-mediated effects on dystrophic degeneration must still be elucidated, these results reinforce the focus on the link between DMD genetic modifiers and immune system modulation. More specifically, these data trigger the question of whether *SPP1* and *CD40* haplotypes significantly synergize to shift immune cell regulation in response to dystrophic muscle degeneration.

LTBP4 modifies availability of TGFβ and myostatin

Latent TGFβ binding protein 4 (LTBP4) was first identified in 1997 as novel binding protein for TGFβ in the extra-cellular matrix [42]. The role of LTBP4 as genetic modifier of muscular dystrophy was first discovered from an unbiased, genomewide quantitative trait loci analysis in a large cohort of dystrophic mice. Specifically, mice on the 129T2/SvEmsJ

background were found to show a milder phenotype than those on the DBA/2J background. The 129T2/SvEmsJ background carries a protective *LTBP4* allele featuring the insertion of 12 amino acids in the proline-rich hinge region of the LTBP4 protein. In contrast, the DBA/2J background and a minority of laboratory mouse strains have a deletion of 12 amino acids in this hinge region. The insertion of 12 residues into the hinge region reduces proteolytic cleavage and latent TGFβ release in the muscle, providing the mechanism by which the "protective" allele acts as compared to the "risk" allele. The protective allele significantly correlated with decreased fibrosis and TGFβ levels in dystrophic murine muscles with a mixed DBA/2J-129T2/SvEmsJ genetic background [17].

In humans, four SNPs (rs2303729, rs1131620, rs1051303 and rs10880) create non-synonymous polymorphisms (V194I, T787A, T820A, and T1140M) in the coding region of the human *LTBP4* gene, discriminating two different haplotypes, the VTTT allele (risk) and the IAAM allele (protective). In non-ambulatory DMD patients, homozygous carriers of the IAAM protective *LTBP4* allele lost ambulation significantly later than the VTTT risk allele carriers, following a recessive model for the protective allele. Importantly, when restricting the analysis to patients on a glucocorticoid steroid regimen, the age gap in ambulation loss between the two haplotypes increased to almost two years [43]. The association between the protective allele and prolonged ambulation was confirmed in a multivariate analysis on a multi-center DMD patient cohort [29]. The beneficial association was also reported in the European/European-American sub-cohort from another multi-center DMD natural history study [28].

Recently, LTBP4 has also been suggested to be a genetic modifier of dilated cardiomyopathy in DMD patients. A multi-center, longitudinal study was conducted in DMD patients, where left ventricular ejection fraction and end diastolic volume were reported as cardiac parameters. The *T/T* allele for SNP rs10880 (specifying a methionine at position 1140; part of the protective haplotype) associated with a protective trend and a later onset of DCM in steroid-treated patients [44]. In addition, a retrospective study on DCM risk was conducted on patient groups stratified according to self-identified ethnicity. This study found that the VTTT risk allele associated with increased DCM risk in European Americans, but not African Americans [45]. The two different human alleles of LTBP4 bind TGFβ with different avidity. Specifically, the IAAM protein bound more latent TGFβ than the VTTT allele [45], which would effectively limit the levels of free TGFβ in injured muscle. In addition, LTBP4 can be considered a multi-TGFβ family ligand binding protein, as it also binds and sequesters the latent forms of myostatin and GDF11, a protein highly related to myostatin [46], further enhancing its anti-wasting role in dystrophic muscle.

The role of LTBP4 in tuning a "hyper-TGFβ" state is particularly intriguing, as this pathway regulates both degeneration and regeneration of striated muscle. Unbiased transcriptional profiling of DMD muscle biopsies in presymptomatic and symptomatic individuals revealed a strong induction of the TGFβ pathway in dystrophic muscle [47]. Accordingly, transcriptional profiling of regenerating muscle revealed *TGFβ1* among the top differentially expressed ligands during muscle regeneration [48, 49]. The TGFβ pathway promotes expansion of fibroblasts and myofiber replacement by fibrotic tissue through a feed-forward cycle that relies on, among others, *miR-21* [50] and *periostin* [51] upregulation.

Furthermore, it is known that TGFβ upregulation is highly detrimental for activation and regenerative potential of resident myoblasts [52]. However, little is known about the effects of LTBP4 on muscle regeneration.

Thus, LTBP4 modifies progression of dystrophic disease by regulating the availability of latent TGFβ around injured myofibers, thereby controlling fibrosis and regeneration, two key features of dystrophic muscles (Figure 1). The multi-ligand binding property of LTBP4 will require more detailed investigation to elucidate the different roles of these different sites in the heterogeneous field of muscle disease. In addition, additional study is necessary to understand whether LTBP4 plays a role in also other conditions related to muscle wasting and atrophy, such as cachexia.

Jagged1: a novel genetic modifier

Jagged1 encodes the trans-membrane ligand of Notch receptors. Jagged1-Notch signaling can be either cell-extrinsic or cell-intrinsic, as both ligand and receptor are transmembrane and the Notch receptor is activated upon physical interaction with its ligands. Activation of the Notch receptor by its ligands, including Jagged1, results in receptor cleavage and migration of its intracellular domain to the nucleus, where it exerts transcriptional regulation in combination with tissue-specific binding partners [53].

Jagged1 has recently been implicated as a genetic modifier of muscular dystrophy by means of whole genome sequencing of dystrophic dogs with variable outcomes. The study compared two “escaper” animals that carried the same loss-of-function dystrophin mutation as other dystrophic dogs. However, the escaper animals had normal lifespan and mild muscle degeneration compared to related dystrophic dogs derived from the same colony. Moreover, progeny from the escaper animals demonstrated transmission of the protective effect to subsequent generations consistent with a genetically-mediated protective effect. Whole genome sequencing data were integrated with muscle transcriptional profiling to uncover a spontaneously occurring mutation in the promoter of *Jagged1*, just upstream of a CpG island. The mutation introduced a myogenin-responsive element, thereby increasing *Jagged1* expression levels within the muscle of the escaper dogs. Furthermore, overexpression of *Jagged1* in a zebrafish model of dystrophin deficiency resulted in rescue of the phenotype [15].

Jagged1 upregulation in muscle may ameliorate dystrophic progression through several means. First, muscle regenerative cells from escaper dogs showed greater proliferative capacity [15]. Interestingly, *Jagged1* is not expressed in quiescent satellite cells, but is rapidly activated upon cell activation [54]. Accordingly, Notch signaling is a potent regulator of muscle regeneration and is gradually lost in aging muscles [55]. In addition, Notch signaling regulation finely controls not only quiescence and activation of satellite cells [56], but also myogenic ability and engraftment of other stem cells, such as mesenchymal stem cells [57] and resident pericytes [58]. However, the precise signaling pathway linking *Jagged1* to its effects on resident myoblasts is still not fully known. Moreover, the question of when *Jagged1* upregulation must occur to appropriately expand the stem cell pool, e.g. during fetal development versus after birth, is still open.

However, Jagged1 may exert its effects on myofibers and myofibroblasts as well. In agreement with this hypothesis, Notch and TGFβ pathways are probably linked by complex crosstalk dynamics. The Jagged1-Notch pathway was found sufficient to inhibit TGFβ signaling and fibrotic potential of cardiac fibroblasts [59]. In addition, Notch activation blocks TGFβ signaling via *Smad7* upregulation in epithelial stem cells [60]. However, how these pathways intertwine in dystrophic muscles must still be comprehensively assessed. Moreover, it will be important to identify which mechanisms are cell-intrinsic or –extrinsic, and in the latter case, to discriminate the cellular compartments associated with ligand presentation and signaling response.

Genetic modifiers in the context of novel drug development

Identification of genetic modifiers can be useful to identify novel pharmacological targets or pathways to counteract dystrophic progression. However, the basic-to-translational path generally hinges on articulated knowledge of mechanisms and the context in which these target modifiers act. Development of novel medicinal products specifically targeting those pathways in dystrophic muscles will require a greater understanding of the upstream and downstream cascades in each of these pathways with focus on intersecting points in their regulatory pathways. Overall, the genetic data support that upregulating *Jagged1* and downregulating osteopontin may be beneficial to the dystrophic muscle, although timing and extent of a putative induced regulation must still be thoroughly addressed as therapeutic strategies for DMD. In the case of Jagged1, this upregulation would ideally be muscle targeted to avoid toxic effects from potentially engaging this pathway in extra-muscle tissues. In the case of osteopontin, a body-wide, constitutive deletion of osteopontin was effective in the *mdx* mouse [14], suggesting that cell types beyond muscle may be critical for mediating its effect.

Investigational approaches to neutralizing TGFβ and myostatin (Figure 2) signaling have captured significant attention for their translational potential [61]. Several approaches are currently being considered as avenues to diminish TGFβ signaling in muscular dystrophy and other muscle wasting disorders. In addition, indirect approaches rely, among others, on modulation of LTBP4. Upregulation of the protective *LTBP4* allele in dystrophic myofibers improved performance and partially corrected histopathology of dystrophin-deficient murine muscles [46]. Although indirect, LTBP4-involving strategies have the advantage of encompassing the latent forms of all TGFβ ligand isoforms and potentially myostatin. However, whether this holds true in DMD patient muscles must still be appropriately addressed.

More direct strategies include monoclonal antibodies targeting TGFβ ligands and the inhibitor of the TGFβ receptor kinase, a major effector of the activated TGFβ receptor. Short-term injection of anti-TGFβ monoclonal antibody in the *mdx* model effectively reduced fibrosis in the diaphragm muscle, a major muscle of respiration and one muscle that shows profound histopathological findings in the *mdx* mouse [62]. However, a significant increase in $CD4^+$ lymphocytes was concomitantly observed in antibody injected *mdx* mice [62]. A clinical-grade anti-TGFβ antibody, fresolimumab, is being evaluated in clinical trials for pulmonary fibrosis, systemic sclerosis, and cancer with promising results [63]. TGFβ

receptor kinase inhibitor Ki26894 is a small molecule, suitable for oral administration, and partially restores weakness and regenerative potential in a murine model of muscle wasting [64]. However, both strategies presently lack clinical studies designed to target and assess skeletal muscle remodeling. Furthermore, the long-term effects of interfering with TGFβ on normal immune tolerance, especially on the homeostasis of regulatory lymphocytes, must be assessed.

Among novel therapeutic strategies targeting TGFβ family ligands, targeting myostatin is arguably the most advanced for muscle disease. Myostatin is a TGFβ family member that negatively regulates muscle mass, and genetic ablation of myostatin attenuates dystrophic progression in *mdx* mice [65]. A monoclonal antibody against the mature ligand form was shown to have beneficial effects on muscle mass and strength of dystrophic mice [66]. However, the role of myostatin in dystrophic degeneration is probably nuanced, as a recent study in dystrophic dogs found that heterozygous ablation of myostatin resulted in disproportionate effects on muscle size and, ultimately, in worsening of the condition [67]. Furthermore, genetic ablation of myostatin in non-dystrophic mice resulted in smaller tendons, which presented a decrease in both fibroblast density and expression of type I collagen, as likely results of decreased p38-Smad2/3 cascade activation [68]. These results warrant caution on strategies for unbalanced and profound myostatin inhibition in dystrophic muscles.

Clinical-grade anti-myostatin monoclonal antibodies are currently being tested in clinical trials. Specifically, Regeneron (Tarrytown, NY) is developing a fully humanized antibody (REGN1033; study #NCT01963598), which has been tested in a randomized, double-blind, placebo-controlled, multicenter phase-II study as a subcutaneous formulation in patients with sarcopenia, although results are still pending. Eli Lilly (Indianapolis, IN) has completed phase-II studies with a humanized antibody (LY2495655) in cancer patients with cachexia (#NCT01505530), also with yet undisclosed results. Intriguingly, a previous phase-II study testing the same antibody in elderly subjects with muscle wasting showed a significant increase in lean muscle mass and partial restoration of muscle power [69]. In addition, Pfizer (New York City, NY) is testing a humanized antibody (PF-06252616; study #NCT02310763) and is currently recruiting DMD patients for a phase-II study. Finally, Scholar Rock (Cambridge, MA) is conducting preclinical evaluation of a monoclonal inhibitory antibody targeting the latent form of myostatin (SRK-015), although its specifications and indications are still undisclosed.

An alternative approach relies on blocking the interaction between myostatin and its receptor, particularly the Activin receptor type IIb (ActRIIb). One such strategy is the ligand trap, namely a soluble, immunoglobulin-hybrid of the extracellular portion of the receptor. The ligand trap competitively sequesters myostatin, reducing its downstream signaling in myofibers. Systemic delivery of anti-myostatin ligand trap induced functional improvement and injury reduction in *mdx* skeletal muscle [70]. Acceleron Pharma (Cambridge, MA) is developing the clinical-grade formulation of a soluble myostatin receptor (ACE-083), currently assessing its safety and tolerability in a phase-I study (#NCT02257489). The same company is also in preclinical development of a multi-GDF ligand trap (ACE-2494), although its indications and plans for further implementation are not yet disclosed.

Inhibitory monoclonal antibodies against the ActRIIb receptor represent a related strategy. Anti-receptor antibodies proved efficacious in slowing muscle mass loss in mice and patients with cachexia [71]. The clinical-grade formulation of anti-ActRIIb antibody (BYM338), developed by Novartis (Basel, Switzerland), has proven effective in increasing lean muscle mass and strength in a randomized, placebo-controlled trial on a limited number (14) of patients with sporadic inclusion body myositis [72]. However, it was subsequently revealed that the phase 2b/3 clinical trial of BYM388 in patients with sporadic inclusion body myositis did not meet its primary endpoint [73]. Nonetheless, the antibody is still being tested on aging-associated sarcopenia in a multi-center study (#NCT01601600), but results are still pending.

Furthermore, an increasing body of evidence links the glucocorticoid steroids to the TGFβ-myostatin circuitry. Glucocorticoid steroids effectively delay loss of ambulation in DMD patients [74–79]. This effect is further increased in the presence of protective *SPP1* and *LTBP4* polymorphisms. Although the primary focus of the study of Bello and colleagues was to investigate the effect size of *SPP1* and *LTBP4* polymorphisms on DMD progression, Cox regression analysis indicated that steroids associated with delayed ambulation loss of up to 0.7 years in addition to the protective effect of *SPP1* rs28357094 SNP (*T/T*), and up to 1.0 year in addition to the protective *LTBP4* haplotype [28]. Similarly, the van den Bergen study showed that steroid use associated with a significant delay in ambulation loss regardless of genotype association study [29]. With respect to *SPP1* and *LTBP4* polymorphisms, steroids showed no significant interaction with the protective *SPP1* SNP, while the additive effect was significant in patients with the protective *LTBP4* haplotype. Furthermore, glucocorticoid steroids seemingly play a role in delaying or alleviating dilated cardiomyopathy (DCM) in DMD patients [80–83]. However, this notion is debated, as studies in mouse models suggest a detrimental role of chronic steroid administration in dystrophic hearts [84–86]. The question of whether genetic modifiers play a role in these divergent effects is still open. In a genetic association study focused on DCM in DMD patients, treatment with steroids did not have a significant independent effect on DCM onset. However, only in steroid-treated patients did the *LTBP4* rs10880 SNP (*T/T*) reach statistical significance in protecting DMD patients from DCM onset [44].

With regards to mechanistic effects on TGFβ/myostatin signaling, glucocorticoid steroids are known to reduce TGFβ levels in *mdx* skeletal muscle [87]. However, chronic dosing of these steroids has a wide array of negative effects, including myostatin activation and consequently muscle atrophy [88]. Considering that glucocorticoid steroids are presently standard of care for DMD patients, it might be possible to combine myostatin inhibition with steroid regimens in order to harness the beneficial effects of glucocorticoid treatment, while hampering the loss in muscle mass. Furthermore, glucocorticoid regimens may synergize with those genetic modifiers that decrease the TGFβ-myostatin cascades, as with the protective *LTBP4* haplotype [29]. However, a deeper understanding of the mechanisms linking glucocorticoid steroids to TGFβ-myostatin cascades in dystrophic myofibers is required to substantiate these hypotheses, to refine current steroid regimens for DMD patients, and to integrate glucocorticoid steroids with pharmacological TGFβ reduction.

Conclusions and future perspectives: breaking the loop of dystrophic progression

In summary, a growing body of evidence is delineating genetic modifiers that regulate the feed-forward loop of muscle wasting and fibrosis that are hallmarks of DMD pathological progression. TGFβ and myostatin cascades mechanistically converge in the promotion of fibrosis and loss of muscle mass, while simultaneously impairing repair and regeneration. It is important to address the question of how osteopontin contributes to the TGFβ-promoted degenerative loop to potentially indicate yet another valuable avenue for pharmacological treatment of dystrophic remodeling. Hypothetically, osteopontin and TGFβ may reinforce each other thereby accelerating the injury-inflammation-fibrosis loop. Analogously, the compelling results obtained from genomic screening of dystrophic dogs corroborate mechanistic studies on the role of *Jagged1* in dystrophic myofiber damage.

These pathways provide unique opportunities for development and testing of novel medicinal products to combat degeneration and fibrosis. Detailed knowledge of genetic modifiers in a wide variety of muscle conditions undoubtedly moves the translational field forward, as is the case for the TGFβ family ligands. Currently, post-hoc analyses according to modifying haplotypes are already recommended by FDA guidelines for drug clinical trials in DMD [89]. Careful assessment of numerous clinical parameters in current clinical trials will yield valuable results to validate or further tailor investigational products for DMD treatment. Importantly, comprehensive strategies integrating diverse approaches and effects will most likely produce the highest curative value in clinical settings, particularly in light of the high genetic and clinical heterogeneity of dystrophic patients.

In conclusion, seminal identification and mechanistic understanding of genetic modifiers are re-shaping our knowledge of muscular dystrophy and significantly priming the therapeutic quest for this yet incurable disease.

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Highlights

- Genetic modifiers change the course of Duchenne Muscular Dystrophy (DMD)
- Genetic modifiers were identified in humans and mice with muscular dystrophy
- LTBP4 modifies muscular dystrophy in mice and humans with muscular dystrophy
- Osteopontin modifies muscular dystrophy
- Jagged1 changes the course of muscular dystrophy

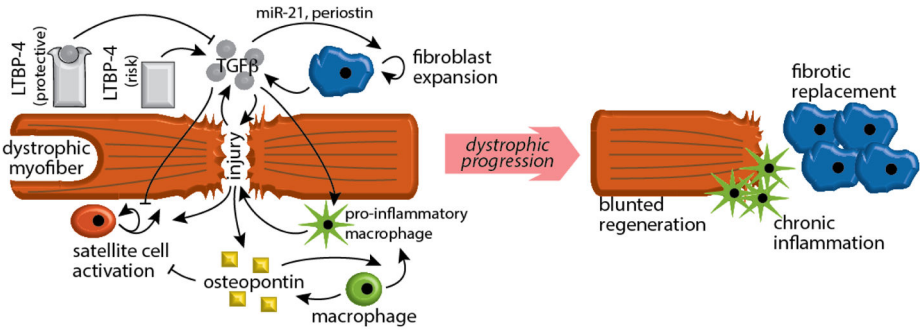

Figure 1. Osteopontin and LTBP4 modify dystrophic progression

Upon chronic myofiber injury, both osteopontin and LTBP4 have the potential to direct dystrophic remodeling via regulation of susceptibility to injury, fibrosis, satellite cell potential and inflammation.

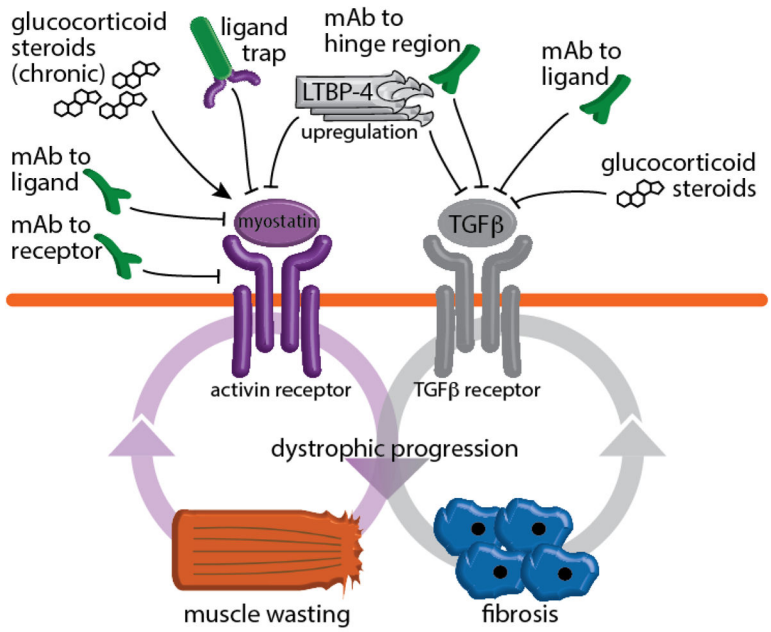

Figure 2. Pharmacological strategies to reduce muscle wasting and fibrosis in dystrophic muscles

TGFβ and myostatin cascades and their intersection with muscle wasting and fibrosis. A number of investigational drugs are currently being tested for reducing both signaling pathways. Glucocorticoid steroids, currently used in DMD treatment, have opposite effects on TGFβ and myostatin activation.