Fibrosis development in early-onset muscular dystrophies: mechanisms and translational implications

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ABSTRACT

Duchenne muscular dystrophy (DMD) is one of the most devastating neuromuscular genetic diseases caused by the absence of dystrophin. The continuous episodes of muscle degeneration and regeneration in dystrophic muscle are accompanied by chronic inflammation and fibrosis deposition, which exacerbate disease progression. Thus, in addition of investigating strategies to cure the primary defect by gene/cell therapeutic strategies, increasing efforts are being placed on identifying the causes of the substitution of muscle by non-functional fibrotic tissue in DMD, aiming to attenuate its severity. Congenital muscular dystrophies (CMDs) are early-onset diseases in which muscle fibrosis is also present. Here we review the emerging findings on the mechanisms that underlie fibrogenesis in muscular dystrophies, and potential anti-fibrotic treatments.

Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is a progressive neuromuscular disorder linked to the X chromosome. It is a highly aggressive, fatal myopathy, with an early onset, that affects about 1 in 3,500 live male births, being the most common inherited muscle disease of childhood. The afflicted boys have an average life span not extending beyond 25 years of age. There is currently no cure for DMD, so that boys with DMD only can be offered palliative care measures to prolong survival [1-3]. The degenerative nature of the disease means that a strategy of reversal of the process is highly unlikely to succeed; rather, a successful intervention is more likely to occur by blocking the pathogenic mechanisms. This requires an in-depth understanding of the molecular pathways, during both normal function and in misregulated situations.

DMD is caused by mutations in the dystrophin gene that prevent functional dystrophin protein from being produced (giving rise to either mutated or shortened versions of the protein) [4]. Dystrophin is a large structural protein that stabilizes the muscle fiber sarcolemma. Without it, fibers become vulnerable to contractions and undergo cycles of necrosis and repair until muscle is replaced by fat and fibrous tissue. DMD also involves failure of the muscle stem cells (also called satellite cells), which normally are quiescent and activate in response to injury to further proliferate, differentiate and fuse to form new muscle fibers, or self-renew to reconstitute the quiescent stem cell pool. However, in DMD, the constant cycles of degeneration-regeneration are thought to exhaust the satellite cell pool over time. Very interestingly, recent studies have shown that, in addition to myofibers, satellite cells express dystrophin and loss of this protein alters the regenerative capacity of these cells in dystrophic muscles, supporting the assumption of exacerbated muscle wasting in DMD by impaired regeneration owing to intrinsic satellite cell dysfunction [5].

DMD is characterized by extensive fibrosis, which is an excessive deposition of extracellular matrix (ECM) components leading to loss of tissue function due to changes in the quality and/or amount of the components of this ECM [6]. It also involves abnormal repair processes, comprising chronic cycles of myofiber necrosis and repair with a sustained infiltration of mononuclear cells in muscle tissue. The causes of fibrous tissue deposition are not well understood. Nevertheless, accumulating evidence unravels inflammation as a driver of fibrosis, with several cell types contributing to ECM

accumulation. In this review, we will discuss the recent advances on this fibrogenic axis in DMD and in congenital muscular dystrophies (CMDs).

The EMC lifecycle

Under normal growth and repair conditions, ECM components are deposited to provide a structural scaffold for new tissue. Several growth factors promote ECM deposition, including transforming growth factor beta (TGFβ), connective tissue growth factor (CTGF), and the renin-angiotensin system (RAS). In addition to these molecules, normal muscle repair also requires factors that regulate the proteolytic degradation of the ECM during regeneration for fiber growth.

Degradation of the ECM involves the large family of matrix metalloproteinases (MMPs), which are calcium-dependent zinc-containing proteolytic enzymes. Degradation is required for facilitating migration of myogenic, inflammatory, vascular and fibroblastic cells to damaged tissue. MMPs are tightly controlled not only by their expression and release but also at the activation step. In damaged muscle, MMPs are mainly released by infiltrating cells, after which they must be activated by proteolytic cleavage of the inactive precursors, in concert with their corresponding inhibitors. The diverse MMP family includes collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9) stromelysins (MMP-3, MMP-7, MMP-10 and MMP-11), membrane-type metalloproteinases (MMP-14 to -17, MMP-24, and MMP-25), and metalloelastase MMP-12 [7]. MMP signaling is essential in muscle regeneration, as demonstrated by the detrimental effect of their inhibition on satellite cell function [8]. The plasminogen activation (PA) system is an enzymatic extracellular cascade that degrades fibrin (among other molecules). The PA system is necessary for matrix turnover and cell migration during tissue repair. The zimogen plasminogen is converted into the active enzyme, plasmin, by two plasminogen activators (PAs): tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). Inhibitors of the PA system include the plasminogen activator inhibitor 1 (PAI-1) and alpha2-antiplasmin, which operate at the level of the PAs or plasmin, respectively [9]. The MMP and PA systems can interact and amplify or synergize their activities to mediate ECM remodeling during tissue repair. Both the MMPs and the PA proteolytic systems have been implicated in the regulation of inflammation,

fibrosis and myogenesis during the muscle regeneration process after injury and in muscular dystrophy (see below).

Fibrosis and its molecular effectors in DMD

Fibrosis impairs function and reduces the overall amount of muscle tissue, and results from chronic degeneration and impaired regeneration of affected myofibers in DMD dystrophic muscle with no functional dystrophin protein. Changes in how satellite cells interact with their surrounding environment can delay muscle repair and alter regeneration and inflammation, thereby accelerating disease progression and fibrosis development.

One of the most potent fibrogenic factors is TGFβ [10, 11] (see Figure 1). This is initially generated as a latent precursor of one of the three isoforms: TGFβ1, TGFβ2, and TGFβ3 [12]. Latent TGFβ is stored in the ECM and is activated by tissue damage or specific growth signals (reviewed in [13, 14]). Activated TGFβ binds to a heterodimeric complex comprised of a TGFβ type I receptor molecule (also called activin linked kinase 5, or ALK5) and a TGFβ type II receptor. TGFβ plays an important regulatory role in regenerating muscle after injury and can also be produced by infiltrating inflammatory, mesenchymal, and tissue-specific cells (reviewed in [15, 16]). It is also activated in the dystrophic muscle of boys with DMD and in *mdx* mice (the mouse model of DMD) [12, 17-19].

Activated TGFβ stimulates fibroblasts to produce ECM proteins, such as collagen and fibronectin. Signaling through the canonical TGFβ pathway in normal fibroblasts starts with ALK5, which phosphorylates the transcription factors Smad2 and -3, allowing these to bind the co-Smad Smad4. This Smad complex is translocated to the nucleus, where it activates transcription of profibrotic genes [20, 21]. A genetic mutation that reduced Smad signaling in transgenic mice improved skeletal muscle and cardiac function in the context of dystrophy-increased TGFβ [22]. TGFβ may also signal through other intracellular transducers besides Smad2/3, such as the Ras/MEK/ERK pathway, the p38 MAPK pathway, the c-abl pathway and JNK, which then work as intracellular signaling mediators [11, 23]. Through these alternative signaling pathways, gene expression can be modified in a promoter-selective fashion, as these transducers are required in divergent processes such as collagen type I expression, ECM contraction (resulting from the mechanical forces

exerted by fibroblasts on the surrounding ECM), and myofibroblast differentiation [24].

The activity of TGFβ signaling can also be altered in skeletal muscle by misregulation of other interfering pathways, with detrimental results. For instance, decreasing the insulin-like growth factor (IGF) signaling in IGF-1R(+/-) heterozygous mice resulted in impaired muscle regeneration, reduced expression of MyoD and myogenin, and increased expression of TGFβ1, α-smooth muscle actin (α-SMA), and collagen I, and ultimately, in fibrosis [25]. In myoblasts, treating with IGF-1 inhibited TGFβ1-stimulated Smad3 phosphorylation and increased phosphorylated-AKT (P-AKT)–Smad3 interactions, which blocked the nuclear translocation of Smad3 and in turn reduced the expression of fibrotic genes. Conversely, reducing IGF-1R levels led to diminished levels of P-AKT, which allowed Smad3 to dissociate and be transported to the nucleus, thereby enhancing the TGFβ1 signaling pathway and fibrosis [25].

TGFβ can also promote fibrosis by modulating the ECM-degrading enzymes. Specifically, increased levels of activated TGFβ lead to reduced production of ECM-degrading enzymes as well as to increased production of their inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitor type-1 (PAI-1). Injecting recombinant TGFβ into skeletal muscle *in vivo* stimulates TGFβ expression by myogenic cells and in other cell types in an autocrine fashion and induces fibrotic tissue accumulation in the area of the injection [19, 26]. Further, skeletal myogenic cells overexpressing TGFβ can differentiate into myofibroblast cells after intramuscular transplantation [26], and this can be blocked by the small leucine-rich proteoglycan decorin, which binds to and inhibits TGFβ (see review [27]). Conversion of satellite cells into fibrogenic cells by the action of TGFβ has also been shown in vitro and in vivo (in injured and in dystrophic muscle) [19, 26]. Transgenic mice overexpressing TGFβ1 in a muscle-specific manner likewise showed muscle wasting and endomysial fibrosis [28]. Recently, it has been proposed that TGFβ-dependent atrophy requires NOX-induced reactive oxygen species (ROS) in skeletal muscle [29]. These data are in agreement with the findings on a transgenic mouse model in which a dominant-negative truncation mutant form of TGFβ type II receptor is expressed in myofibers [30]. Transgene expression mitigated the dystrophic phenotype of δ-sarcoglycan-null mice, reduced damage and improved injury-induced muscle regeneration. Mechanistically, transgenic mice displayed reduced fragility of the myofiber plasma membrane, which was attributed to the induction of antioxidant proteins and increased protection against ROS accumulation, indicating that the deleterious effects of TGFβ in

skeletal muscle are also mediated by the myofibers [30]. Intriguingly, fibrosis in *mdx* mice can be modulated with the latent TGFβ-binding protein 4 (LTBP4), which, through proteolytic cleavage, regulates the release and bioavailability of TGFβ from the ECM, pointing to LTBP4 as a potential target to regulate TGFβ signaling and modify the progression of muscular dystrophy [31]. Indeed, further studies using transgenic mice carrying the human LTBP4 gene in the mdx background revealed augmented TGFβ release by increased proteolytic susceptibility, exacerbated inflammatory infiltration, fibrosis and muscle weakness [32]. These results support the possible use of antibodies to block LTBP4 cleavage as a strategy to reduce fibrosis and wasting in DMD.

Similar to TGFβ, CTGF also affects muscular dystrophies by directly inducing fibrotic processes and inhibiting myogenesis [33]. CTGF, a non-structural regulatory protein present in the ECM, has been found to be elevated in skeletal muscle from DMD patients, dystrophic dogs and mdx mice [34]. Importantly, in mdx mice, fibrosis development can be reduced with an antibody that blocks CTGF [35]. Crosstalk with TGFβ allows CTGF to amplify TGFβ effects on fibrosis. Studies in myoblasts have shown that certain phospholipids, such as lysophosphatidic acid (LPA), synergize with TGFβ to stimulate CTGF expression [36]. Moreover, TGFβ-mediated upregulation of CTGF relies on the cooperative actions of Smad and Sp1/Sp3 transcription factors [37] and sphingosine kinase-1/Sp3 signaling has been implicated on the profibrotic effects of CTGF [38]. In fibroblasts, CTGF can induce collagen type 1, α5 integrin, and fibronectin more effectively than TGFβ [39], and CTGF overexpression in muscle of mice induces strong fibrosis [40]. Decorin, which is down-regulated in DMD muscle biopsies [41] appears to negatively regulate the activity of CTGF (as well as of TGFβ), most likely by directly binding to CTGF, while CTGF induces the expression of decorin in a potential autoregulation [42]. Thus, TGFβ and CTGF have deleterious effects on muscular dystrophy by blocking muscle repair and promoting fibrosis [30, 33].

The RAS system, involving renin-angiotensin, principally associated to the regulation of blood pressure, has been found activated in DMD [43] (see Figure 2). Upon inhibition of RAS by blockade of angiotensin-converting enzyme (ACE) via enalapril, the expression of CTGF was reduced in dystrophic muscle [44]. This is in agreement with higher levels of ACE in muscles of DMD patients. Angiotensin 1 (Ang 1) arises from conversion of Angiotensin by renin, and is further converted into angiotensin 2 (Ang 2) by ACE, which binds Ang 2 receptor type 1 (AT1) and type 2 (AT2) on the cell surface, to transmit

intracellular signaling. The pro-fibrotic action of Ang 2 is well documented [45, 46], whereas Ang-(1-7), a small derivative of Ang 2, has mainly an anti-fibrotic activity. Dystrophic mice treated with Ang-(1-7) showed reduced muscle fibrosis and pathology, and these effects involved attenuation of the TGFβ-Smad pathway, and reduction of expression of downstream effectors, such as the pro-fibrotic microRNA (miR)-21 (see below) [18, 47]. miR-21 was upregulated in muscle biopsies of DMD patients and correlated with the expression of collagen, supporting pharmacological modulation of this microRNA for antifibrotic therapies [48]. The action of Ang-(1-7) is initiated thought binding to its receptor Mas. In agreement with this, administration of the Mas antagonist (A-779) or genetic deletion of the Mas receptor in the mdx mice leads to increased TGFβ signaling, fibrosis and worsening of the dystrophic phenotype [47]. Interestingly, the protein levels and the activity of the enzyme ACE2 (an homologue of ACE) were found to be increased in the sarcolemma of the myofibers in fibrotic muscles. Paradoxically, further increase in ACE2 activity by artificial overexpression resulted in a reduction of fibrosis and macrophage infiltration in vivo, leading to the interpretation that this enzyme may function as an inflammatory/fibrotic sensor in skeletal muscle which acts as a compensatory mechanism to increase Ang-(1-7) production decreasing fibrosis development [49]. In vitro experiments have further shown that increased TGFβ signaling (but not CTGF or Ang 2) in muscle-derived fibroblast reduces Mas receptor expression, an effect which was not observed in myogenic cells or differentiated myotubes [50]. This effect may lead functionally to an increased fibrotic phenotype by dystrophic fibroblasts since the reduction of Mas receptor makes them less sensitive to Ang-(1-7) stimulation. Besides stimulating fibrosis, Ang 2 effects in muscle have also been associated with the promotion of myofiber atrophy through activation of the ubiquitin ligases atrogin-1 and MuRF-1, whereas Ang-(1-7)-Mas counteracts these effects and activates growth promoting AKT signaling. Overall, these data suggest that a potential therapeutical use of Ang-(1-7) to improve muscle wasting and fibrosis in pathologies in which the levels of Ang 2 are elevated [51].

A concerted action of the PA system, involved in muscle tissue remodeling, and the expression of the pro-fibrotic miR-21 was established in dystrophic muscle through regulation of TGFβ activity by the uPA/PAI-1 proteolytic balance [18]. Mdx mice deficient in PAI-1 had increased muscle fibrosis through unrestricted uPA-induced proteolytic cleavage of TGFβ, which impacted on fibroblast activation. Hyperactivation of TGFβ leads to increased expression of mature miR-21, through a non-genomic Smad2/3 action, which

resulted in inhibition of the miR-21 target PTEN (a phosphatase that inhibits the growth promoting kinase AKT). As a consequence, in the absence of PAI-1, excessive miR-21 leads to increased and persistent levels of AKT in fibroblasts, which resulted in increased proliferation and matrix deposition in dystrophic muscle. Fibrosis in muscles of old mdx mice, or young mdx mice deficient in PAI-1, was reduced via an antagomiR against miR-21, as well as by interfering with uPA proteolytic activity. Conversely, ectopic expression of miR-21 advanced fibrosis in young dystrophic muscle. Because antagonizing miR-21 and uPA-mediated proteolysis could attenuate fibrosis progression in aged *mdx* mice (with highly fibrotic muscles), both strategies could be taken into account for combating human DMD fibrosis at advanced disease stages [18, 48].

Macrophages as regulators of fibrosis in skeletal muscle

Neutrophils and monocytes/macrophages are the inflammatory cell types that first infiltrate the injured skeletal muscle, having a major role both in phagocitosing debris as in stimulating myogenesis during tissue repair ([52], reviewed in [53, 54]). Thus, as in other tissues, altered inflammation is known to cause defective repair and excessive matrix deposition, both in injured and dystrophic muscle (see [15, 55, 56] for review). For instance, genetic deletion of toll-like receptor 4 (TLR4), which responds to certain endogenous host molecules associated with tissue damage, reduced inflammatory gene expression, macrophage infiltration and fibrosis in *mdx* mice improving dystrophy [57].

Fibrosis is often associated with chronic inflammation. Supporting this link, in dystrophic muscle, TGFβ is mainly produced by macrophages, and this promotes matrix deposition [12]. Macrophages conform a heterogeneous cellular population and exert distinct functions. In contrast to “classically-activated” M1 pro-inflammatory macrophages, “alternatively-activated” M2 anti-inflammatory macrophages have been proposed as promoters of fibrotic conditions [58-60]. Th2 cytokines (IL-4 and IL-13) induce M2 macrophage activation, and interestingly, this pathway has been associated to production of arginase I, an enzyme involved in collagen synthesis [61]. M2 macrophages (producing arginase) are found in fibrotic areas of muscle in *mdx* mice and DMD patients, including cardiac muscle, strengthening the possible profibrotic function of M2 macrophages in DMD [17, 62-65].

In addition to providing proliferative support for satellite cells, M1 macrophages also sustain chronic inflammation if persisting over time, and therefore contribute to fibrogenesis. Persistent activation of M1 macrophages can be induced by ECM molecules deposited in dystrophic muscle, such as fibrinogen, which binds Mac-1 integrin on macrophages ([17, 66] reviewed in [67]). Loss of fibrinogen in *mdx* mice therefore can reduce muscle inflammation and fibrosis [17]. More specifically, transgenic *mdx* mice expressing a mutated form of fibrinogen, incapable of binding Mac-1, exhibited reduced inflammation and fibrosis, and improved muscle regeneration [68]. It is worth highlighting the exquisite timing required for M1 and M2 macrophage persistence in order to induce the proliferation and apoptosis of fibro-adipogenic progenitors (FAPs) at the required phase during muscle regeneration. Sequential TNFα and TGFβ production by M1 and M2 macrophages, respectively, regulates the fate of FAPs, the precursor cells for fibroblasts (i.e. collagen-producing cells) and adipogenic cells in injured and dystrophic muscle [69-71], so that altered balance of these cytokines leads to FAPs persistence and fibrosis development [72]. From the clinical point of view, imatinib and nilotinib (both interfering with FAPs activation) have been shown to reduce muscle fibrosis in a DMD mouse model [72, 73]. An alternative pathway whereby FAPs can be activated in injured muscle is through eosinophil-produced Th2 cytokines, thus implicating this immune cell type in the process of muscle regeneration [74]. A role for eosinophils in promoting fibrosis development in dystrophic muscle has also been proposed [75].

Cellular sources of fibrosis

Several lines of evidence support FAPs as the major precursors of fibrogenic cells in dystrophic muscle [69, 70]. These cells, expressing the platelet-derived growth factor receptor PDGFRα, can give trophic support to satellite cells as well as produce a transient ECM during the early stages of the muscle repair process. However, if persisting over time, they can have a deleterious role in this process. Indeed, their elevated number in dystrophic muscle has been associated with high presence of fibroblasts and increased collagen production, thus exacerbating muscular dystrophy [72, 76].

In fibrotic pathologies affecting kidney or heart, several cell types can give origin to fibroblasts, through cellular transdifferentiation ([56, 77-79]). In muscle, recent studies

have shown that satellite cells, as well as cells of endothelial or hematopoietic origin, can undergo a fibrogenic conversion and produce high levels of matrix proteins while losing their identifying gene expression program. These cellular conversions have been proposed to contribute to the reduced regeneration capacity of skeletal muscle as well as to the increased fibrosis in dystrophic muscle [19, 80]. Macrophage deletion has also been shown to promote endothelial to mesenchymal transition in injured muscle and to contribute to fibrosis [81]. Of interest, a subset of pericyte cells producing Adam12 has also been shown to contribute to fibrosis in injured muscle [82]. Morever, elimination of these Adam12 positive fraction reduced collagen deposition after muscle injury, whereas ectopic expression of Adam12 exacerbated fibrosis in mdx muscle [82]. Thus, despite the predominant role of FAPs as origins of fibrosis in injured and dystrophic skeletal muscle, additional cell types contribute to collagen deposition, particularly in chronically damaged muscle with sustained inflammation.

Potential therapeutic approaches to reduce fibrosis in DMD

A core aim of gene or cell therapy for DMD is to replace the defective or missing dystrophin gene, thereby curing the disease by targeting the cause of muscle degeneration. Strategies for this include viral replacement therapy, plasmid-mediated nonviral gene expression, stem cell transplantation, antisense oligonucleotide–induced exon skipping, and nonsense mutation suppression by drugs; these strategies however have not yet been successful [83-86]. A very important step towards the therapeutic use of gene editing techniques for DMD treatment has recently been achieved in *mdx* mice, by using adeno-associated virus for CRISPR–Cas9 delivery to skeletal and cardiac muscle [87-89]. These studies demonstrate the possibility of restoring dystrophin expression in muscles of adult dystrophic mice within the range of expected therapeutic beneficial effects in humans. Importantly, satellite cells can also be targeted with this technique, assuring the availability of a pool of modified muscle stem cells which express dystrophin [88]. These results are very promising in spite of the need to investigate further the possibility of off-target effects and eventual undesired immunological reactions. In any case, adjunct therapies aimed at preventing fibrosis progression should be considered essential, as fibrosis development reduces the amount of target tissue available for repair and directly decreases quality of life and lifespan of dystrophic patients [3]. Treatments aimed at reducing the presence of

fibrotic cells or inhibiting fibrogenic molecules are being tested in preclinical mouse models at present.

Several components of the RAS system (discussed above), such as AT2 and ACE, have been used as targets to decrease dystrophic muscle fibrosis in animal models, with promising results. Losartan, which is antagonistic to AT2 signaling and which is commonly used to treat high blood pressure, improves muscle strength and ameliorates fibrosis in dy(2J)/dy(2J) mice with laminin-α2-deficient congenital muscular dystrophy [90]. Mechanistically, this drug inhibits TGFβ and MAPK signaling and shifts the nuclear factor kappa B (NFκB) signaling pathway favoring survival versus apoptosis [91]. Losartan treatment also improved muscle function in aged mice, diminished fibrosis in injured muscle of mice and in hind limb immobilization-induced sarcopenia [92-94], and in heart and muscles of *mdx* mice [95, 96]. Moreover, long-term administration to *mdx* mice did not show adverse effects in other organs [97]. Lisinopril is an ACE-inhibitor that preserves cardiac and skeletal muscle integrity in *mdx* mice [98]. Indeed, because of the positive pre-clinical effects shown for losartan and lisinopril, a double-blind randomized clinical trial to compare lisinopril versus losartan for the treatment of cardiomyopathy in human DMD patients has been performed showing comparable positive effects for both drugs [99]. Similarly to lisinopril, administration of the ACE inhibitor enalapril to *mdx* mice decreased skeletal muscle fibrosis [44].

Antifibrotic strategies being presently tested also include the inhibition of autophagy [100] and targeting of growth factors and cytokines that promote matrix deposition. Neutralizing antibodies against TGFβ and CTGF were shown to reduce fibrosis in *mdx* mice [35, 101]. Similarly, interference with PDGFRα signaling using imatinib and nilotinib also decreased muscle fibrosis and attenuated disease progression [72, 102, 103]. Other antifibrotic molecules have also been tested, including halofuginone, which ameliorated muscular dystrophy in mice by reducing fibrosis, up-regulating utrophyn and promoting satellite cell survival, likely in combination with an anti-inflammatory actions [104-108]. The promising results obtained with halofuginone have led to the initiation of a clinical trial with DMD boys (reference NCT01847573 http://clinicaltrials.gov/show/NCT01847573) which is under evaluation.

Congenital muscular dystrophies, fibrosis and ECM remodeling

Congenital muscular dystrophies (CMDs) comprise a highly heterogeneous group of early-onset muscle disorders with great clinical and genetic heterogeneity (see reviews in [109, 110]). At difference with DMD, in which the cytoplasm protein dystrophin is truncated/absent, they can be caused by alterations of proteins in different locations which can be grouped in two major categories:

- ECM structural proteins, basal lamina proteins or receptors for ECM components. This group includes mutations of each of the three Collagen VI genes (COL6A1, COL6A2 and COL6A3) causing Ulrich Congenital Muscular Dystrophy (UCMD), Bethlem Myopathy (BM), congenital myosclerosis and limb-girdle muscular dystrophy [111]; laminin α2 (LAMA2-related muscular dystrophy) [112] and integrin α7 [113] mutations and alterations of the glycosylation of α-dystroglycan [114] causing α-dystroglycan related dystrophy.
- Intracellular and nuclear proteins. This group comprises nuclear envelope proteins such as lamin A/C (LMNA gene), causing LMNA-related dystrophy [115]; SEPN1 related myopathy, by mutations of the SEPN1 gene [116] (which encodes selenoprotein (SelN), primarily involved in oxidation-reduction reactions and located at the endoplasmic reticulum) and mutations of the RYR1 gene [117], coding for the sarcoplasmic reticulum calcium release channel (Recessive RYR1-related myopathy with CMD presentation).

Since CMDs are rare diseases, specific information concerning the pathogenesis of fibrosis on these complex and heterogeneous diseases is restricted. Nevertheless the presence of fibrosis and fat infiltration has been reported in muscle biopsies of patients, frequently with endomysial location, and sometimes accompanied by the presence of inflammatory infiltrates and signs of degeneration and regeneration in congenital muscular dystrophies related with alterations of COL6, laminin α2, α-dystroglycan, SEPN1 and RYR1 and in LMNA [109].

CMDs caused by collagen VI and laminin α2 mutations have been studied more deeply than other forms of CMD. The finding that collagen VI is not made by myocytes but by the resident interstitial muscle fibroblast population [118] suggests a “non-cell autonomous" nature of collagen VI-related muscle fiber disorders, postulating a role for fibroblasts in inducing myofiber degeneration by modifying the ECM composition. Collagen VI is

present at the basement membrane around muscle cells [119], where it is supposed to participate in the maintenance of their functional integrity and in tissue remodeling. The α-6 chain of collagen VI accumulates in dystrophic compared to normal muscles; this correlates with the extracellular accumulation of the α-6 chain in response to TGFβ1 stimulation of primary human muscle cells undergoing myofibroblast conversion [120], supporting a role for collagen VI in fibrotic ECM remodeling. Consistently with these findings, a beneficial effect of collagen VI depletion in muscle pathology and functionality in the context of muscle dystrophy has been proposed [121]. Indeed, intercrossing the mouse model of limb-girdle muscular dystrophy type 2C γ-sarcoglycan-null mice (which displays prominent fibrosis and increased ECM collagen VI deposition in muscles) with a Col6a2-deficient mouse model lead to a reduction of fibrosis in double mutant mice but, surprisingly, no improvements of muscle function [121]. These results, while confirming the role of collagen VI in the regulation of fibrotic pathology, also indicate that the correct presence of the protein is necessary for myofiber homeostasis, in agreement with the pathological findings in muscles of collagen VI mutant mice [121-124]. Mechanistically, several pathways have been found altered in collagen VI mutant skeletal muscles, as indicated by recent proteomic insights, which include changes in the unfolded protein response, hexosamine pathway, and amino acid and fatty acid metabolism, suggesting an association of endoplasmic reticulum (ER) stress, metabolic dysregulation, autophagic impairment, and alteration in mechano-transduction signaling with the pathogenesis of the disease [125]. Collectively, ECM alterations due to collagen VI modifications can lead indirectly to myofiber modifications, including the chronic accumulation of intracellular damaged proteins and organelles, causing autophagic dysregulation and apoptosis. Indeed, autophagy-restoring strategies have been successful in improving myofiber degeneration in collagen VI muscular dystrophies [126]. Interestingly, collagen VI alterations not only induce myofiber damage, but also impair regeneration and satellite cell self-renewal in a non cell-autonomous way since restoration of collagen VI by grafting wild-type fibroblasts into the muscles of Col6a1($^{-/-}$) mice rescued the satellite cell defects and ameliorated the biomechanical properties of diseased muscles [127].

Fibrosis is also a prominent feature of LAMA2-related congenital muscular dystrophy (MDC1A). Similarly to DMD, muscles of MDC1A patients showed decreased decorin and increased TGFβ1 expression levels compared with healthy individuals [41]. In mouse models of both types of dystrophy, up-regulation of the pro-fibrotic miR-21 correlating

with fibrosis development has been reported [18, 128], suggesting, at least partially overlapping fibrogenic mechanisms in DMD and LAMA2-related dystrophy, despite the differences in the primary cause of muscle degeneration. Consistently, as mentioned above, Ang 2 receptor antagonists improve fibrosis and regeneration in mouse models of LAMA2-related congenital muscular dystrophy by reducing TGFβ1 signaling [90, 129]. Similarly to its effects on *mdx* muscles, down modulation of TGFβ1 signaling with the antifibrotic drug halofuginone has positive effects on fibrosis reduction in laminin α2 deficient mice [130]. A common feature of LAMA2-related muscular dystrophy with collagen VI related dystrophies is the increased apoptotic death of myofibers [131-133]; however, whereas a reduction in myofiber autophagic activity has been reported in collagen VI alterations [126], overactivation of the autophagy-lysosome pathway and the ubiquitin-proteasome system have been detected in mouse and human LAMA2- dystrophic muscles [134, 135]. An updated specific review on the pathophysiology of laminin-α2 chain-deficient congenital muscular dystrophy has been recently published [136].

Integrin α7 mutations have also been causally related with CMD. Integrin α7 binds Laminin-2 in skeletal muscle [137] and laminin-2 deficient muscles show reduced integrin α7 subunit expression [138]. Consistently, restoration of α7 integrin expression in laminin-2 deficient muscles alleviated the myopathic phenotype observed in MDC1A [139]. However, integrin α7 subunit genetic deletion did not aggravate the phenotype of laminin α2 chain-deficient mice [140] and integrin α7-deficient muscles in mice show a relatively mild dystrophic phenotype, affecting mainly to the myotendinous junctions [141, 142]. This is in contrast with the effects of the absence of laminin α2 chain in dystrophin or β-sarcoglycan mutant mice that drastically aggravates muscle pathology [143]. These findings highlight the complexity of the functional interactions between transmembrane cellular receptors and proteins at the myomatrix.

The functions of dystroglycan (DG), which is the central component of the dystrophin-glycoprotein complex (DGC) in skeletal muscle, have been studied in several experimental models (see [144] for review). Among them, a knock-in mouse model that carries a missense mutation in DAG1 (T190M) that selectively impairs further modification of phosphorylated O-mannosyl glycans (mediated by the glycosyltransferase LARGE), that is required for high-affinity binding to laminin and that causes primary human dystroglycanopathy was generated [145]. Unexpectedly, in this mouse, the dystrophic phenotype was mild and the presence of prominent fibrosis was not reported.

LMNA related dystrophy is caused by mutations in the lamin A/C (LMNA) gene and are responsible for a range of cardiac and muscular dystrophies (see [115, 146] for recent reviews). Heart abnormalities have deserved comparatively more attention than those of skeletal muscle in this group of diseases. Nevertheless, fibrosis has been reported in both types of striated tissue, correlating with alterations of intracellular signaling pathways, including activation of extracellular signal-regulated kinase1/2 (ERK1/2) MAPK signaling, AKT/mTOR and TGFβ signaling [146] and providing the basis for pharmacological therapeutic interventions [115]. Interestingly, increased levels of activated P-Smad2/3 proteins were observed in the fibrotic areas of mice with mutated LMNA that develop muscular dystrophy and dilated cardiomyopathy resembling human disease [147], indicating overactivation of TGFβ dependent pathways. In agreement with these findings, a mechanisms linking nuclear lamin defects and tissue fibrosis has been proposed [148]. Fibroblast with deficits in lamin A/C functions have increased phosphorylation of retinoblastoma protein (Rb) and Smad2, likely via inhibition of protein phosphatase 2A (PP2A), which binds to lamin A/C and responds to TGFβ. Thus, the consequence of abnormal Smad transcription factor hyper-phosphorylation is increased proliferation and excessive collagen production by fibroblast [148].

Limited amount of information is available concerning SEPN1 and RyR1 related myopathies and fibrogenesis. SEPN1 is a member of the selenocysteine-containing protein family that localizes in the ER lumen and has an ubiquitous distribution in the body. In two different mouse models of SEPN1 deficiency, the muscular phenotype was absent in basal conditions and pathological changes were only observed after inducing oxidative stress by exercise [149, 150] or by forced expression of oxidant enzymes such as ER oxidoreductin 1 (ERO1), a major ER protein disulfide oxidase [151]. Mutations of the skeletal muscle Ca^{2+} release channel RyR1 are associated with several human skeletal muscle disorders. RyR1 mutant mice displayed hypertrophic fibers, disorganized sarcoplasmic myofilaments, multiple internalized nuclei, and increased interstitial connective tissue in skeletal muscles [152]. RyR1 mutations have been linked with dysregulated intracellular Ca^{2+} release, which induces oxidative/nitrosative stress, leading to S-nitrosylation of RyR1, altered mitochondria and increased ROS/RNS in muscle, which may explain some pathological features [153, 154]. Interestingly, the effects of the mutation could be prevented pharmacologically by normalizing RyR1 Ca^{2+} efflux or antioxidant treatments [155]. This later strategy has been employed in clinical trials in SEPN1- and RYR1-related myopathies and in DMD (reviewed in [155]). Taken together, despite that DMD has been studied more

intensively than CMDs, evidence is emerging that the pathogenic pathways leading to fibrogenesis and muscle remodeling may be conserved.

CONCLUSION

Fibrosis is the end stage of an aberrant tissue reparative process, whereby chronic inflammation impacts on matrix-producing cells, leading to excessive ECM accumulation, which in turn substitutes normal by non-functional fibrotic tissue. Muscle fibrosis is a hallmark for DMD. If the fibrotic muscle in DMD patients could be redirected towards regeneration, at least partially, thus enhancing muscle integrity and fitness, the quality of life of patients could be considerably improved. Chronic fibrosis represents also a barrier for restoring dystrophin in DMD muscle through either gene- or cell-based approaches. Thus, reducing fibrosis in DMD is key both to improve muscle function and enhance the chances of success of dystrophin-based therapies. Notably, as fibrogenesis appears to be a common trait of CMDs, similar anti-fibrotic therapeutic strategies could be postulated. Advances in identifying pro-fibrotic growth factors and cytokines and their downstream signaling effectors, as well as new cell types directly contributing to fibrosis progression, have been made, and hopefully will crystallize in finding safe and efficient anti-fibrotic treatments for muscular dystrophies.

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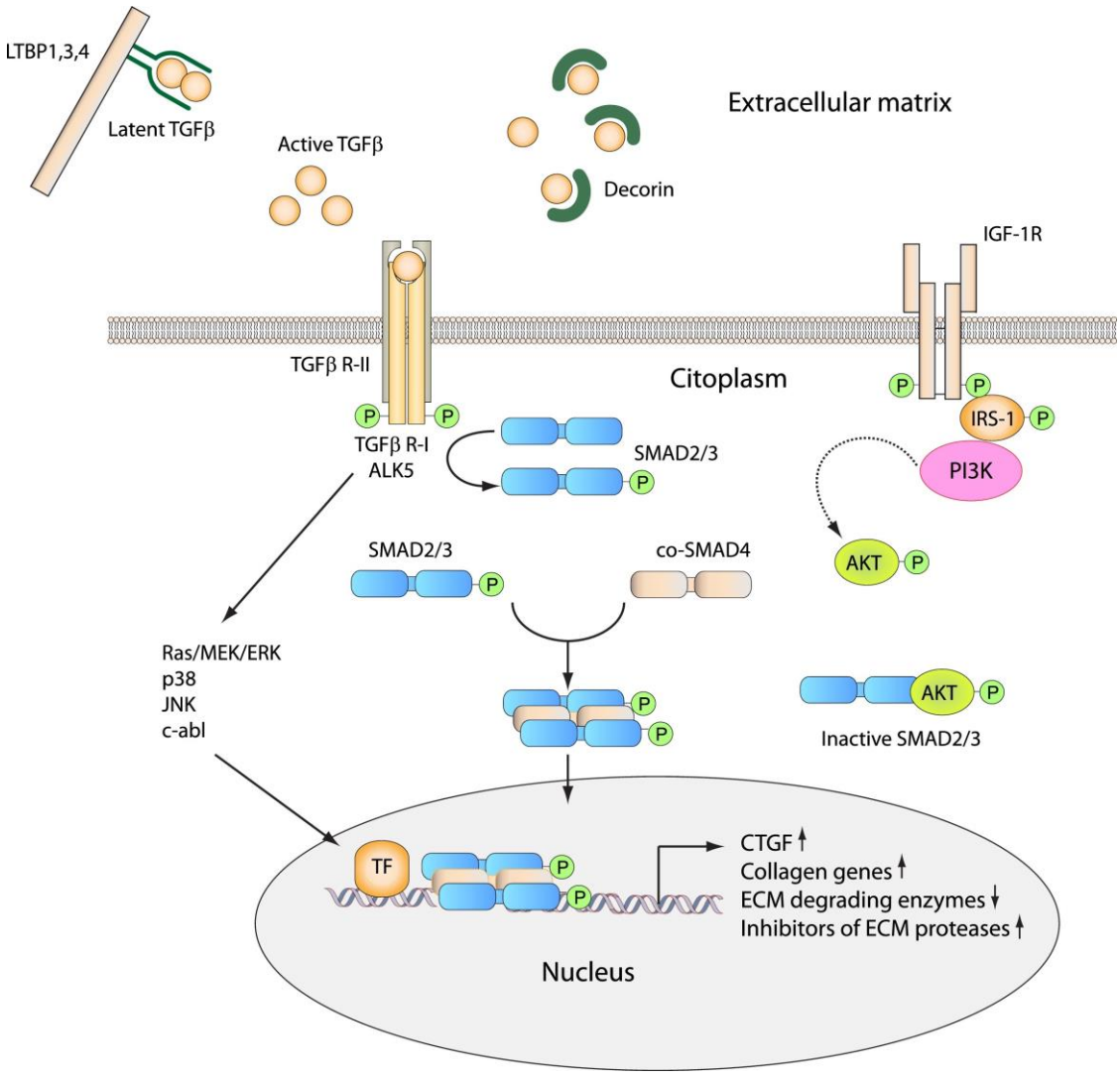

Fig. 1. TGFβ and PI3K/AKT pathways in muscle fibrosis. At the extracellular matrix, TGFβ is present in an inactive form, bound to latent TGFβ binding proteins (LTBP). Once active, TGFβ binds to cell membrane heterodimeric receptor complex formed by TGFβ type I (or ALK5) and type II receptors (TGFβ R-I and TGFβ R-II). Decorin, a protein present in the ECM, can inhibit TGFβ binding to its receptors. In the cytoplasm, TGFβ receptor activation leads to phosphorylation of Smad2/3 transcription factors that upon binding to Smad4 translocate to the nucleus and activate the expression of pro-fibrotic genes in cooperation with other transcription factors. Activation of the insulin-like growth factor receptor (IGF-1R) leads to increased AKT activity. Phosphorylated AKT binds to Smad2/3 and inhibits their nuclear translocation and pro-fibrotic gene transcription.

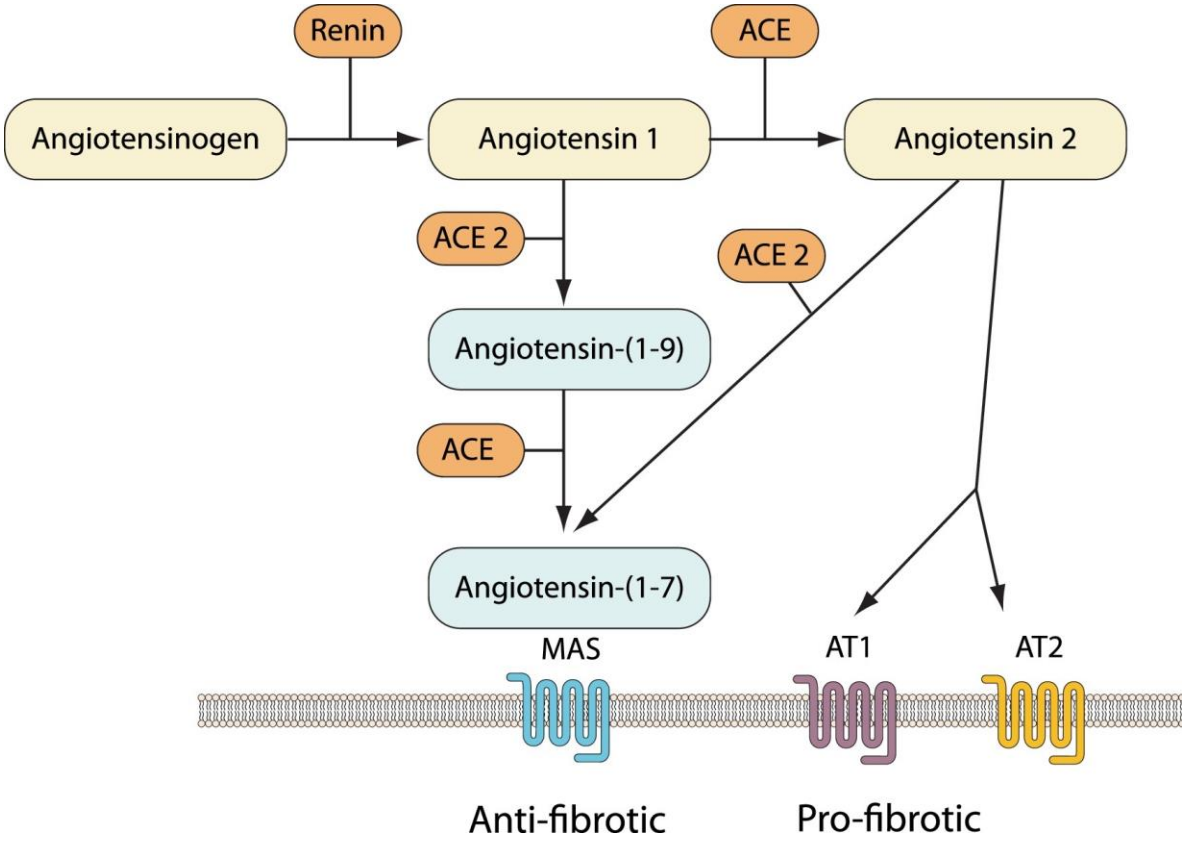

Fig. 2. Components of the renin-angiotensin system (RAS) involved in the development of muscle fibrosis. Renin activity produces Angiotensin 1 (Ang 1) which is transformed into Angiotensin 2 (Ang 2) by the angiotensin-converting enzyme (ACE). Ang 2 interacts with its receptors AT1 and AT2 on the cell surface and induces pro-fibrotic responses in the target cells. Angiotensin-converting enzyme 2 (ACE 2) produces a truncated form of angiotensin called angiotensin-(1-7) (Ang-(1-7)), which has anti-fibrotic effects after binding to Mas receptor.