

Cellular Mechanisms of Tissue Fibrosis. 4. Structural and functional consequences of skeletal muscle fibrosis

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Lieber RL, Ward SR. Cellular Mechanisms of Tissue Fibrosis. 4. Structural and functional consequences of skeletal muscle fibrosis. *Am J Physiol Cell Physiol* 305: C241–C252, 2013. First published June 12, 2013; doi:10.1152/ajpcell.00173.2013.—Skeletal muscle fibrosis can be a devastating clinical problem that arises from many causes, including primary skeletal muscle tissue diseases, as seen in the muscular dystrophies, or it can be secondary to events that include trauma to muscle or brain injury. The cellular source of activated fibroblasts (myofibroblasts) may include resident fibroblasts, adult muscle stem cells, or inflammatory or perivascular cells, depending on the model studied. Even though it is likely that there is no single source for all myofibroblasts, a common mechanism for the production of fibrosis is via the transforming growth factor- β /phosphorylated Smad3 pathway. This pathway and its downstream targets thus provide loci for antifibrotic therapies, as do methods for blocking the transdifferentiation of progenitors into activated fibroblasts. A structural model for the extracellular collagen network of skeletal muscle is needed so that measurements of collagen content, morphology, and gene expression can be related to mechanical properties. Approaches used to study fibrosis in tissues, such as lung, kidney, and liver, need to be applied to studies of skeletal muscle to identify ways to prevent or even cure the devastating maladies of skeletal muscle.

myofibroblast; passive mechanics; muscle mechanics; stiffness

SKELETAL MUSCLE EXTRACELLULAR matrix (ECM) not only plays the general supportive role that is typical of other tissues, such as lung, liver, and heart, but the proper functioning of the skeletal muscle matrix is critical to maintain the normal locomotor ability of an organism. Alterations in the ECM perturb functional properties of muscle in a way that can be directly measured, and, in fact, the health of skeletal muscle, which makes up $\sim 50\%$ of the body mass (74), is tantamount to the health of the organism itself.

In this review, we focus on unique aspects of the structural and functional changes in muscles that undergo fibrosis. While skeletal muscle retains many of the fibrotic mechanisms of other tissues, the functional effects of fibrosis, such as in muscle contractures, in which a joint takes on a permanently fixed position that requires relief by surgery (75), or retracted rotator cuff muscles, which are separated from the bone and are very difficult to reattach (133), can be devastating. Because the organization of skeletal muscle ECM is closely linked to its mechanical function, this tissue provides a unique opportunity to study the mechanics of fibrosis in a way that is not possible with other tissues.

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Skeletal Muscle ECM

Skeletal muscle tissue is dominated by large, multinuclear muscle fibers that extend along the muscle's length. Thus it is perhaps not surprising that the field of skeletal muscle physiology has focused on the properties of these fibers, including their size (44), function (19), gene regulation (108), and development (130), with a surprising paucity of studies that define the structural and functional properties of the ECM. The majority of microscopic studies of skeletal muscle are performed on tissue cross sections, which reveal tightly packed polygonal fibers surrounded by a very small amount of ECM, typically only $\sim 5\%$ (27) (Fig. 1A). While muscle fiber type specialization among mammalian muscles (12, 122) and within muscles (69) is well characterized, the surrounding extracellular tissue has been treated more or less generically. Thus students are typically taught that skeletal muscle fibers are surrounded by endomysial connective tissue, bundles of muscle fibers are surrounded by perimysial connective tissue, and the entire muscle is surrounded by epimysial connective tissue (28). However, inspection of a muscle cross section reveals that these designations are relatively arbitrary (Fig. 1A). Thus, while understanding the cellular mechanics and biology of muscle cells represents one of the triumphs of modern science, there is a lack of understanding of the noncontractile skeletal muscle ECM.

ECM Composition

Numerous noncontractile proteins have been isolated from skeletal muscle homogenates and are believed to make up the ECM. Many of the details of these protein populations have recently been reviewed (38, 77) and are only briefly outlined here. As with most tissues, the major ECM muscle protein is collagen, dominated by the type I and type III isoforms. Muscle cells themselves are surrounded by a basement membrane composed of type IV collagen, which is intimately associated with an integrin focal adhesion complex. This complex includes primarily $\alpha_7\beta_1$ - and other α -integrin subunits, the identity of which depends on the precise developmental time point investigated and whether the integrin is in the region of the sarcolemma (muscle plasma membrane), the muscle-tendon junction, or the neuromuscular junction (81, 107). A second focal adhesion between myofibrils and the ECM is formed by the dystroglycan complex (94), an array of proteins that may perform a role similar to that of integrins; abnormalities of the dystroglycan complex are associated with a large number of primary myopathies (31). The transmission of force between muscle cells and the ECM is not unique to muscle tissue, since

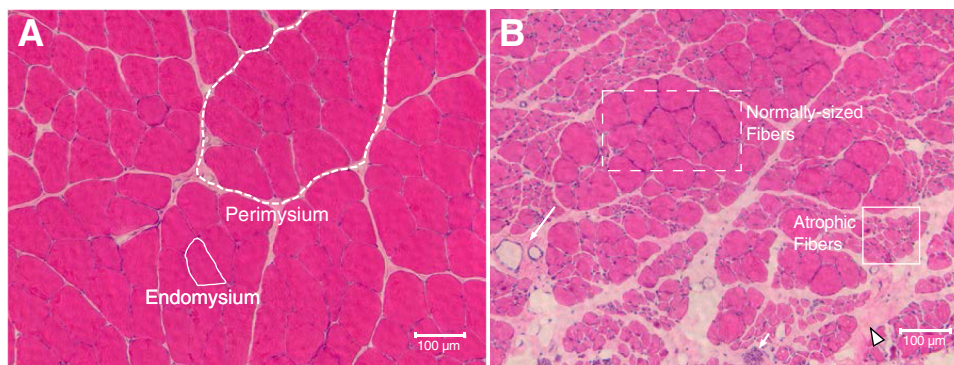


Fig. 1. *A*: micrograph of a cross section of healthy skeletal (rat tibialis anterior) muscle demonstrating normal morphology that consists of tightly packed polygonal fibers with a small amount (~5%) of extracellular material. Traditional location of endomysial connective tissue is outlined with a solid line; perimysial tissue is outlined with a dashed line. However, as can be seen elsewhere in the micrograph, this distinction can be arbitrary. *B*: micrograph of a cross section of skeletal muscle demonstrating fibrotic morphology in which extracellular material is increased to ~20% of the cross section, fibers are loosely packed, extracellular space is hypercellular, and fiber sizes are highly variable. This muscle was injected twice with botulinum toxin type A (Botox, Allergan; 6 U/kg in 100 μ l) at a 3-mo interval and tested after 6 mo.

it likely occurs in all cells but, in skeletal muscle, is essentially its primary function (97).

The intimate association between the extracellular and intracellular milieus creates a tissue that acts as an excellent mechanotransducer of the environment and is very sensitive to alterations in use patterns. Many of these “altered-use” patterns have profound clinical relevance and are active areas of study. These include muscle hypertrophy due to exercise, stretch, and hormonal treatment, as well as muscle atrophy due to tenotomy, spaceflight, aging, spinal cord injury, denervation, and regeneration. Each of these models is discussed in detail elsewhere (74, 98, 102, 109). Nearly all these altered-use models result in muscle fibrosis. Unlike the generally opposite direction of muscle fiber adaptation to increased use (where muscle fibers hypertrophy and decrease speed) compared with decreased use (where muscle fibers atrophy and increase speed), almost all altered-use patterns result in increased skeletal muscle ECM (Fig. 1*B*).

Extracellular Collagen Matrix Structure

The structure of the extracellular collagen network of skeletal muscle is poorly understood. Apart from the classic mathematical models and vivid images of the endomysial connective tissue network published decades ago by Trotter and colleagues (100, 123, 124), there are almost no other quantitative physiological or mechanical studies of skeletal muscle ECM that permit a mechanistic understanding of the way in which skeletal muscles bear passive tension. One recent development in this area relates to the rediscovery of the so-called perimysial cables in skeletal muscles that were originally described by Borg and Caulfield (10). The highly organized longitudinal nature of these cables (Fig. 2*A*) identifies them as likely candidates for major passive load-bearing structures in skeletal muscles. These cables are composed of collagen fibrils that are stereotypically arranged in bundles of 25–100 fibrils and occupy the region between muscle fibers and fiber bundles (Fig. 2, *B* and *C*). These cables can traverse extremely long distances (>150 μ m) within the muscle ECM and provide a parallel elastic element to the muscle tissue itself (38). The extent to which loading is borne inside and outside the muscle cell is not known.

Measures of Skeletal Muscle Fibrosis

An important and unresolved issue regarding skeletal muscle fibrosis is the definition of “muscle fibrosis.” As a general rule, fibrosis is characterized by abnormal accumulation of ECM, but time and severity of disease are important contributors to fibrosis. For example, accumulation of ECM can be seen in nearly all models of muscle injury or damage, but this is often transient and thought to stabilize the contractile apparatus while normal adaptive or regenerative processes proceed. In contrast, long-term accumulation of ECM interferes with function, does not resolve under normal physiological conditions, and is therefore considered an end-stage process. To be consistent with other tissues, skeletal muscle fibrosis could be defined as an abnormal and unresolvable, chronic increase in extracellular connective tissue that interferes with function.

The precise quantification of skeletal muscle fibrosis can be difficult. This is due in part to the fact that, as mentioned above, a deterministic quantitative model of collagen arrangement for muscle ECM is not available. Additionally, situations in which fibrosis occurs (e.g., exercise-induced injury, severe atrophy, chronic inflammation, and dystrophies) dramatically alter muscle fiber size, which complicates expressing fibrosis as a “number” or even a relative fraction. Typically, skeletal muscle assays quantify the cross-sectional area fraction of ECM by excluding muscle fibers using image-processing algorithms and then report the amount of ECM as “area fraction,” as suggested in stereology (128). The problem with this approach is that if muscle fibers atrophy and ECM structure remains the same, ECM will occupy a greater fraction of the muscle cross section. In addition, this approach gives no information regarding collagen isoforms and cross-linking, which can also affect function. For normal muscle, the ECM area fraction is typically ~5%, but this value can increase dramatically in diseased or injured states. In addition to increased fractional area (area fraction) of ECM in fibrotic muscles, because the pathological response often includes fiber degeneration and regeneration, muscle fibrosis is also accompanied by a large increase in muscle fiber size variation. Thus pathologists often highlight the increase in the area fraction of ECM, as well as the increased fiber size variability, as the

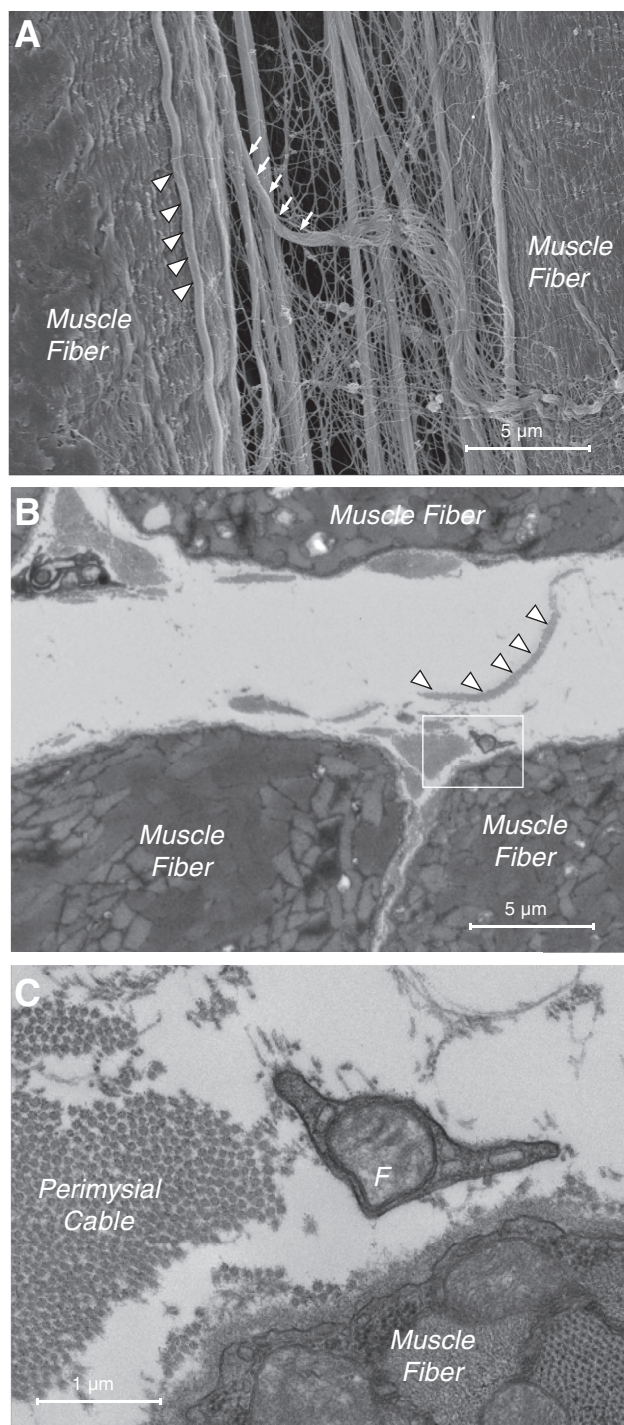


Fig. 2. A: scanning electron micrograph of perimysial cables in a stretched mouse extensor digitorum longus (EDL) muscle. These cables run longitudinally along the length of the muscle fiber without noticeable connections to the fiber itself over $\sim 100\text{-}\mu\text{m}$ distances (arrowheads). Cables consist of bundles of collagen fibrils, as can be seen in the cable that has frayed (arrows). (Micrograph modified from Ref. 38.) B: transmission electron micrograph of wild-type mouse EDL muscle in the extracellular space region. A perimysial cable is demarcated with the box, and a wide ribbonlike perimysial cable is marked with arrowheads. C: higher-magnification view of the region in B indicates that the perimysial cable is composed of collagen fibrils. (This is most easily seen on longitudinal sections that demonstrate the characteristic striation pattern of type I collagen fibrils.) Also shown are superficial aspects of the muscle cell and a section of an extracellular-resident fibroblast (F). (Micrographs courtesy of Allison Gillies.)

defining factor in describing a tissue sample as pathological (16, 23).

Skeletal muscle fibrosis can also be expressed in terms of the total amount of collagen present in the tissue, as measured by the content of hydroxyproline, a major component of collagen derived from hydroxylation of the amino acid proline by prolyl oxidase. While this assay has been used for decades (48), expression of collagen mass relative to a known muscle protein is only rarely reported. In the majority of studies of skeletal muscle, collagen contents (typically expressed as micrograms of collagen per wet or dry muscle mass) of experimental and control groups are compared. While this content provides some insight into a tissue's response to treatment, in the few cases where collagen content has been quantified along with skeletal muscle mechanical stiffness, the two values show only a weak correlation (Fig. 3). Thus the method used to quantify fibrosis in skeletal muscle will depend on whether one uses a morphological assay such as area fraction, a biochemical assay such as collagen content, or a functional assay such as stiffness. It is not possible to quantitatively interchange results of these assays, although they usually change in the same direction. Thus a muscle with increased collagen content also typically has an increased stiffness, but a quantitative relationship between the two has not been established.

Muscle Function and Fibrosis

Because fibrosis occurs in the ECM, functional measurements of fibrosis can be made in the absence of muscle fiber activation and muscle contraction. It has been known for centuries that skeletal muscles bear load when passively lengthened (8), but a full understanding of the origin of passive skeletal muscle tension is lacking. This vagueness is in stark contrast to the highly detailed molecular description of active muscle sarcomere force generation elucidated almost 50 years ago (26, 46) and recent descriptions of the intracellular myofibrillar passive load bearing via the molecule titin (49, 92, 93). Passively elongated muscle cells typically bear stresses of ~ 50 kPa (units of kPa are used to express stress, or force per unit area: $1\text{ Pa} = 1\text{ N/m}^2$; biological tissues typically bear stresses in the kilo- to megapascal range; Table 1). Passively elongated bundles of muscle cells (that contain ECM as well as fibers)

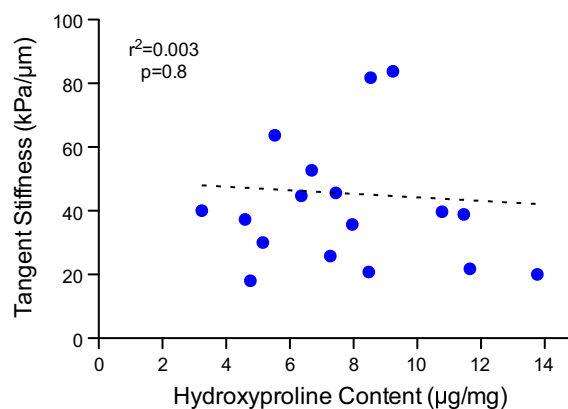


Fig. 3. Correlation between collagen content as measured by hydroxyproline assay and mechanical tangent stiffness measured biomechanically in human muscle fiber bundles, as described in Ref. 111. Note that collagen content alone is a very poor predictor of bundle stiffness.

Table 1. *Tensile modulus values for various connective tissues*

| Tissue | Nominal Tensile Modulus | Sample Reference |
|-----------|-------------------------|------------------|
| Muscle | 20–100 kPa | 91 |
| Tendon | 1–1.5 GPa | 14 |
| Ligament | 1.2–2.0 GPa | 35 |
| Cartilage | 0.4–0.9 GPa | 2 |
| Bone | 10–20 GPa | 43 |

bear stresses on the order of 250 kPa. Comparisons of fiber and bundle stresses can be difficult, as the method of cross-sectional area measurement (which is required for the stress calculation) can be subject to error, especially in muscle fibers, which can have highly variable shapes. However, on the basis of the 5- to 25-fold difference in fiber stress compared with bundle stress and the small amount of ECM relative to muscle fibers, modulus values (i.e., the intrinsic stress-bearing ability of ECM) for the ECM of skeletal muscle are relatively high, on the order of hundreds of megapascals, or even in the gigapascal range (37, 76, 100).

To definitively show that the ECM was the major source of passive load bearing, we compared what we called a “group” of muscle fibers (single muscle fibers that had each been dissected free of the ECM and then tied together) with a naturally occurring bundle (the same number of fibers, but in their native configuration, containing both fibers and ECM; Fig. 4A). Stretching each of these samples in small increments and measuring both force and cross-sectional area revealed that the natural muscle fiber bundle had much higher stress, even though it had the same cross-sectional area as the group of fibers (Fig. 4B). Furthermore, the fiber groups bore the same stress (force/area), even though they were 10–20 times larger than the single fibers (86). Given that the ECM in these tissues makes up only 5–10% of the cross-sectional area, an overall muscle modulus of 40 kPa (Fig. 4B) corresponds to an ECM modulus of ~1 GPa. Therefore, while it is clear that the intracellular protein titin bears much of the force in muscle cells (99), from a tissue point of view, the major muscle passive load bearing occurs in the ECM. Current estimates of ECM modulus are 5–25 times the modulus of muscle cells.

Model Systems of Skeletal Muscle Fibrosis

There are numerous models of fibrosis in skeletal muscle (Table 2), probably the most dramatic of which are the muscular dystrophies, which have cyclic degeneration and regeneration. In different types of dystrophy, ECM area fraction increases as much 10-fold, and the associated muscle increases in stiffness. These models are highly complex, since cellular infiltration, muscle atrophy, fiber size variability, and regenerating fibers accompany fibrosis and fill the tissue area (Fig. 5). Since this type of fibrosis is extreme and clinically relevant, studies of muscle fibrosis and of potential therapeutic interventions for its prevention are often performed using dystrophic models (see below).

However, skeletal muscle fibrosis can also occur in settings where the treatment of the muscle is extremely simple and does not involve a large degree of degeneration or regeneration. One approach is to simply stretch a muscle-tendon unit and secure it at a fixed length. This maneuver results in acute muscle

lengthening, addition of serial sarcomeres (116), muscle fiber atrophy, increased collagen content and collagen area fraction (65), and increased muscle stiffness (115). This fibrotic response occurs with negligible muscle fiber regeneration. Thus, while cellular regeneration often accompanies fibrosis, the two are not necessarily causally related. In a recent study, we chronically stretched skeletal muscles (by securing the distal tibialis anterior tendon to the ankle extensor retinaculum) and measured muscle stiffness and sarcomeric properties (115). This protocol dramatically increased muscle stiffness far more than would be expected on the basis of muscle fiber length change alone, and, importantly, the relationship between active and passive muscle force generation was largely disrupted (Fig. 6). Specifically, active force produced by control and stretched muscle was approximately the same (Fig. 6, dotted line), whereas the fibrotic, stretched muscle was much stiffer than the control muscle.

Muscle unloading that results from tenotomy [i.e., cutting the muscle insertion tendon and allowing the muscle to retract (1)] is accompanied by significant muscle fiber atrophy, which is mediated by catabolic processes involving the muscle ubiq-

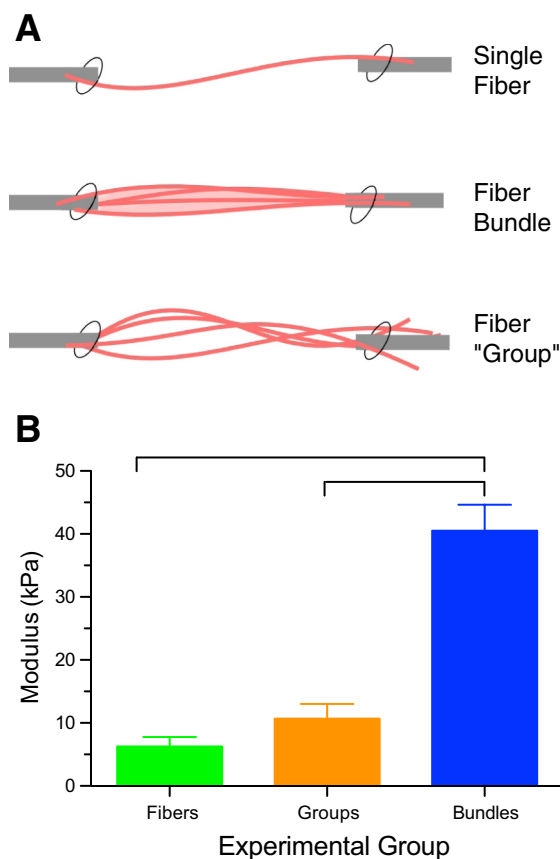


Fig. 4. A: schematic illustration of the arrangement of 3 specimen types. Single fibers (curved pink lines) were isolated from the muscle and tested individually or secured in groups. Bundles of a similar number of fibers embedded in ECM (light pink) were isolated and similarly secured. (Modified from Ref. 86 with permission from Elsevier). B: normalized stiffness of fibers, fiber groups, and fiber bundles. Fiber bundles have a significantly higher modulus than either individual fibers or fiber groups, demonstrating that the ECM provides a large fraction of the load bearing. Fiber and fiber group moduli were not significantly different from each other. Connecting bars represent significant differences ($P < 0.05$). (Data replotted from Ref. 86 with permission from Elsevier.)

Table 2. Models of *in vivo* skeletal muscle fibrosis and common features

| Model | Animal | Muscle | Stiffness | Connective Tissue | Inflammation | MHCs | Myofibroblasts (α -SMA) | Atrophy | Regeneration | Fat | Sample References |
|--------------------------|--------|------------------|-----------|-------------------|--------------|------|---------------------------------|---------|--------------|-----|---------------------|
| Mechanical | | | | | | | | | | | |
| Laceration | Mouse | Gastrocnemius | | I | I | | I/F | I | I | | 17, 34, 71 |
| Puncture | Mouse | TA | I | I | | | I/F | I | I | | 21 |
| Chronic stretch | Rabbit | EDII | I | | | | | NC | | | 115 |
| Immobilization | Mouse | Soleus | I | I | | | | I | | | 114 |
| | Rat | TA | | I | | | | | | | 61 |
| | | Soleus | | I | | | | | | | |
| | | Gastrocnemius | | I | | | | | | | |
| Exercise | Human | Vastus lateralis | | I | | | | | | | 53 |
| Hindlimb suspension | Rat | Soleus | | NC | | I | | I | | | 52, 54, 118 |
| | | | | | | | | | | | |
| Tenotomy | Mouse | Supraspinatus | | | | | | I | | I | 105 |
| | Rat | Supraspinatus | I | I | | I | | I | | I | 5, 39, 127 |
| | Dog | Infraspinatus | I | I | | | | I/F | | I | 104 |
| | Sheep | Infraspinatus | I | I/F | | | | | | I | 85 |
| | Human | Supraspinatus | I | | | | | I | | I | 36, 55 |
| Chemical or toxin | | | | | | | | | | | |
| Cardiotoxin | Mouse | TA | | I | I | | I/F | I | I | | 71 |
| Glycerol | | | | | | | | | | | |
| Botulinum toxin | Rat | | I | | | | | | | | 119 |
| hrTGF- β 1 | Mouse | TA | | I | I | | I/F | I | I | D | 71 |
| BaCl ₂ | Mouse | Gastrocnemius | | I | | | | I | I | | 66 |
| | | TA | | I | | | | I | I | | 66 |
| Genetic | | | | | | | | | | | |
| Desmin KO | Mouse | | I | I | I | S | | I | I | | 71 |
| <i>Mdx</i> | Mouse | Diaphragm | | I | I | I | I/F | I | I | | 58, 60 |
| | | Biceps femoris | | I | I | I | I/F | I | I | | |
| DMD | Human | | I | I | I | I | I/F | I | I | I | 29, 33, 79, 82, 103 |
| Acquired disease | | | | | | | | | | | |
| Cerebral palsy | Human | Semitendinosus | I | I | | S | | | | | 111 |
| | | Gracilis | I | I | | S | | | | | 111 |
| Stroke | Human | Plantarflexors | I | | | F | | I | | | 40, 50 |
| Spinal cord injury | Human | Vastus lateralis | I | I | | F | | | | | 7, 13 |
| Osteoarthritis | Human | Vastus medialis | | I | | | | I | I | | 32 |
| Diabetes (type 2) | Human | | | I | | | | I | I | | |
| Aging | | | | | | | | | | | |
| | Rat | EDL | I | I | | | | | | | 3 |
| | | Soleus | I | I | | | | | | | |
| | Human | Vastus lateralis | | NC | | S | | I | | I | 45, 51, 67 |

MHCs, myosin heavy chains; α -SMA, α -smooth muscle actin; TA, tibialis anterior; EDII, 2nd toe digital extensor; hrTGF- β 1, human recombinant transforming growth factor- β 1; KO, knockout; DMD, Duchenne muscular dystrophy; EDL, extensor digitorum longus; I, increased; D, decreased; S, slower; F, faster; NC, no change. Blank entry indicates no data available.

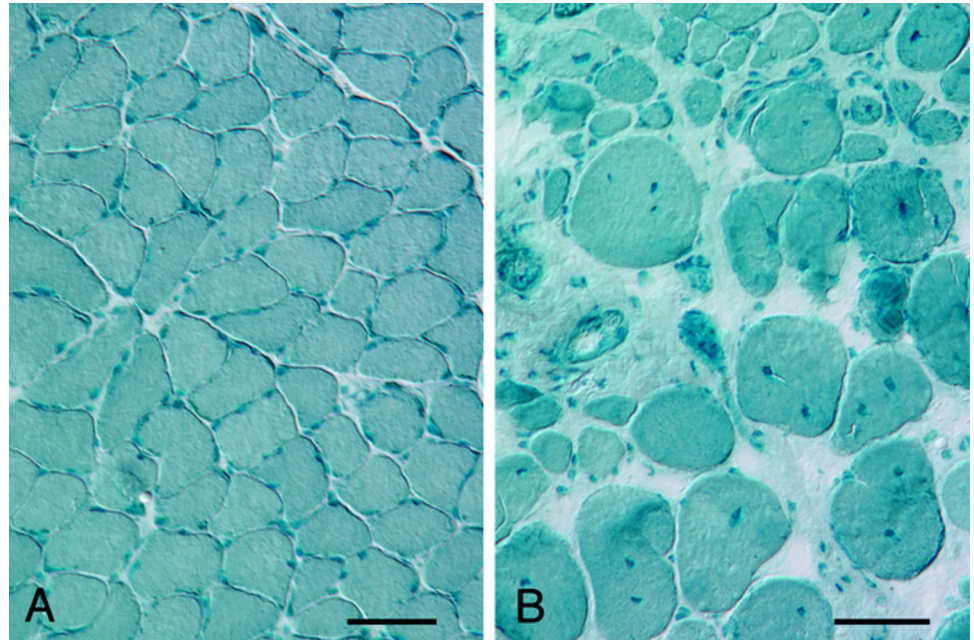
uitin-proteasome pathway (68, 90) and its associated E3 ligase partners (106). This raises the intriguing possibility that muscle fiber atrophy may be associated with fibrosis. Indeed, it is provocative that most fibrotic skeletal muscles are also atrophied or vice versa. It thus appears that resetting a muscle to an altered length initiates the fibrotic process. Whether this is an attempt to restore length or fix length or is simply a secondary response to muscle atrophy is not known.

Another interesting model of skeletal muscle fibrosis has been created with the deletion (by homologous recombination) of the intermediate filament protein desmin (89). Loss of this protein decreases muscle fiber stiffness but also increases ECM stiffness and fibrosis (87). Since the process of fibrosis in this model is relatively slow and involves very little fiber regeneration, we investigated the timing of the change in muscle fiber properties relative to the change in ECM properties in the

absence of regeneration. Measurement of fiber and bundle properties of neonatal and adult mice revealed that muscle fibrosis developed after the fibers changed their properties, which suggests that the biomechanical properties of the muscle cell and its ECM may be mutually sensed. In this way, a change in the ECM may compensate for changes in cellular mechanical properties. This is potentially a system in which the tissue mechanical properties are regulated by communication among the various tissue domains, which has been suggested by others (130).

In an attempt to understand the mechanism of fibrosis in the intermediate filament knockout model, we performed transcriptional profiling of this fibrotic tissue (88) and found that intramuscular inflammation was one of the most dramatically altered systems. Specifically, a group of 18 genes were differentially expressed between desmin-deficient (*Des*^{-/-})

Fig. 5. Micrograph of a cross section of a regenerated skeletal muscle from a control mouse (A) and a mouse model of Duchenne muscular dystrophy, the *mdx* mouse (B). In contrast with the control muscle and the muscle shown in Fig. 1, the *mdx* muscle has more loosely packed fibers of highly varying size with a large amount (>25%) of hypercellular extracellular material. Scale bars, 60 μ m. (From Ref. 121.)



and wild-type (WT) samples (Fig. 7), suggesting that inflammation contributes to *Des*^{-/-} muscle ECM remodeling. Consistent with these results, gene ontology (GO) pathways involved in inflammation and response to stress, including response to wounding (GO:0009611), inflammatory response (GO:0006954), and regulation of tumor necrosis factor (GO:0032680), were overrepresented in the *Des*^{-/-} compared with the WT muscle. Since tissue inflammation in general (131) and skeletal muscle inflammation in particular (129) are associated with fibrosis, this provides another potential tissue-level explanation for the fibrotic response. Moreover, inflammatory cells can be a source of cytokines that lead to fibrotic tissue (see below).

Cellular and Molecular Basis of Skeletal Muscle Fibrosis

Not only are the structural and functional properties of the skeletal muscle ECM poorly understood, but also there is a lack of insight regarding mechanisms that lead to fibrosis, at

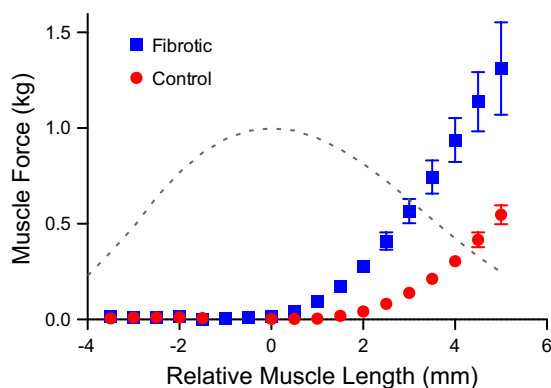


Fig. 6. Active (dotted lines) and passive (red and blue symbols) length-tension curves of a control rabbit muscle and a rabbit muscle subjected to chronic stretch at a fixed length (Fibrotic). Chronic surgical stretch results in a leftward shift in passive mechanical properties, demonstrating the functional mechanical effects of fibrosis. (Data replotted from Ref. 115.)

least relative to those previously reported in this series (15, 41, 131). While functional effects of fibrosis and many of its morphological signs have been well studied in skeletal muscle, the cellular and molecular determinants of fibrosis are less well defined for skeletal muscle than for other tissues (note that skeletal muscle is not among the tissues listed in Table 1 of Ref. 129). The majority of the studies in this area involve muscular dystrophies, a hallmark of which is skeletal muscle fibrosis, as noted above.

Transforming Growth Factor- β in Skeletal Muscle Fibrosis

The cellular signaling processes that dominate skeletal muscle fibrosis follow the general pattern for other tissues, as previously discussed in this series of articles (15, 41, 131). Of particular importance is the cytokine transforming growth factor- β (TGF β), which, in skeletal muscle, takes the form of TGF β 1 (80). TGF β 1 is released from injured muscle fibers or, in an autocrine fashion, from fibers exposed to TGF β 1 (71) and accompanies muscle inflammation (78), whereby inflammatory cells can deposit this cytokine. TGF β receptor activation leads to phosphorylation of receptor-regulated Smad (R-Smad), and, in turn, transcriptional pathways are activated or repressed (22). The quantity and type of Smad partners determine which genes will be targeted and if they will be activated or repressed. Variations in the expression of TGF β receptors, the presence of receptor antagonists, and the variety of mechanisms downstream of Smad explain why TGF β can be involved in muscle fibrosis, as well as other processes, such as cell proliferation, differentiation, cancer, and immunity (80). Because of the central role of TGF β 1 in skeletal muscle fibrosis, anti-TGF β 1 therapy is a strategy used to minimize or ameliorate the effects of fibrosis (34, 132).

Myostatin in Skeletal Muscle Fibrosis

Another member of the TGF β superfamily and a muscle-specific factor is myostatin, also known as growth differentia-

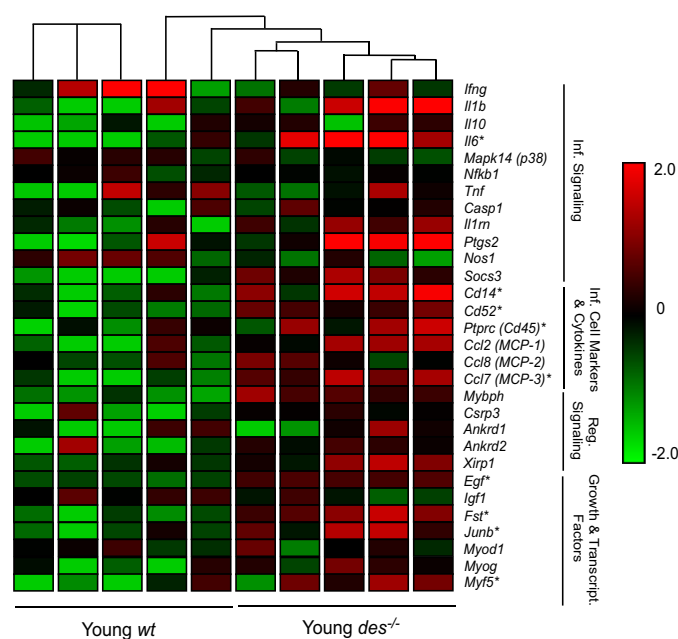


Fig. 7. Data from a transcriptional profile of control muscles [Young wild-type (wt), $n = 4$] and muscles with a desmin intermediate filament deletion [desmin-deficient (Des^{-/-}), $n = 4$]. Des^{-/-} muscle demonstrates increased ECM-specific gene expression, as shown in the normalized gene expression pattern for 42 genes involved in ECM structure and maintenance. Expression levels are shown on a color scale, with green and red representing low and high expression, respectively. Hierarchical clustering is represented by connecting lines at the top of the grid, with lines closest to the grid denoting the most similar samples. Des^{-/-} muscle samples have a distinct expression pattern of ECM genes compared with wild-type muscles, showing a higher expression of the majority of the listed genes indicated by the red color scheme. ECM-related genes are subdivided into 4 categories: basement membrane, ECM constituents, proteases, and protease inhibitors. Inf, inflammatory; Reg, regulatory. *Significantly higher expression values, as determined by 2-way ANOVA. (Data from Ref. 87.)

tion factor 8 (83). Myostatin has a well-known role in regulating muscle mass: animals lacking the myostatin gene (84) or humans with mutated myostatin proteins (110) have extremely large muscles. Skeletal muscle regenerates more effectively and develops less fibrosis in the absence of myostatin or in the presence of myostatin inhibitors (132). The dystrophic *mdx* mouse model has less fibrosis and increased muscle size in the absence of myostatin, suggesting synergistic actions between myostatin and other fibrotic pathways (126), apparently via apoptotic mechanisms (9). Myostatin can directly stimulate muscle fibroblasts to proliferate and expresses ECM proteins (73). In the latter experiments, myostatin stimulated proliferation of fibroblasts from dystrophic muscle and increased the resistance of fibroblasts to apoptosis.

ANG II in Skeletal Muscle Fibrosis

While anti-TGF β -based therapies have proven effective in animal models, clinical application is hampered by severe side effects, lack of oral dosing profiles, and regulatory hurdles. In this regard, fibrosis occurs in the presence of the peptide hormone ANG II, which also causes vasoconstriction and increases blood pressure. Because blood pressure regulation is a well-studied area of cardiovascular physiology and of major clinical significance, there is interest in using agents that block actions of ANG as an antifibrotic therapy. ANG II, which is

derived from the action of ANG II-converting enzyme (ACE) on ANG I, acts on smooth muscle (and other types of) cells via ANG receptors. Importantly, the use of ACE inhibitors and ANG receptor blockers decreases fibrosis in heart, liver, kidney, lung, and muscle (6). Patients treated with ANG-modulating drugs can display decreased muscle atrophy and muscle tissue adipogenesis (96), implying that antifibrotic therapy has positive effects on muscle health. However, no change in muscle properties were observed in experiments in which rats were treated with ACE inhibitors for 12 wk (4), so it is not clear if modulation of ANG directly affects muscle cells.

Collagen Triple Helix Repeat-Containing 1 Protein in Skeletal Muscle Fibrosis

A fascinating recent development in the muscular dystrophy field is the increased expression of collagen triple helix repeat-containing 1 (Cthrc1) protein in response to TGF β 1/Smad3 activation. Cthrc1 was first identified as a unique transcript in balloon-injured arteries, along with deposition of collagen and the presence of myofibroblasts (101). Increased Cthrc1 expression has also been associated with increased cell migration, motility, and invasion in cancer models (117) and, importantly, with inhibition of TGF β 1-stimulated collagen I synthesis (70). These findings suggest that tissue matrix remodeling and repair could be affected by Cthrc1 by limiting collagen I synthesis and promoting cell migration, with Cthrc1 playing a part in the physiological machinery that reverses the fibrotic reaction after termination of the TGF β stimulus. Moreover, where collagen I and Cthrc1 colocalized, the collagen fibers appeared smaller, suggesting that Cthrc1 is also involved in collagen turnover. In the dystrophic mouse models, infiltrating myofibroblasts were the source of the Cthrc1. Cthrc1 is thus another attractive therapeutic target to prevent fibrosis.

Wnt Signaling in Skeletal Muscle Fibrosis

Similar to the relationship between muscle atrophy and fibrosis is the relationship between muscle aging and fibrosis (42). Using a surgical model in which the circulatory systems of older and younger mice are connected, Brack et al. (11) demonstrated that systemic factors are involved in the aging process, which results in increased collagen deposition in muscles. Since it had been observed that the Wnt pathway participates in fibrogenic conversion of pulmonary cells (18) and liver cells (62), this pathway was tested in the skeletal muscle aging model. Intriguingly, it was determined that not only was the Wnt pathway involved (since use of the specific Wnt inhibitors Dickkopf homolog 1 and secreted frizzled-related protein 3 reversed the effect), but the cells that became fibrogenic (as indicated by the ER-TR7 marker) were those that had been myogenic, as demonstrated using lineage tracing. In the older animals, myogenic cells (identified on the basis of their expression of Pax7) were more likely to move toward fibrogenesis than myogenesis. The extent to which myogenic precursors are involved in other models of fibrogenesis is not known. Other components of the Wnt signaling cascade, including glycogen synthase kinase 3 β and its substrate β -catenin, which translocates to the muscle cell nucleus upon activation, were also involved in this process. Because the Wnt signaling pathway is so highly conserved across species, these

experiments suggest that the fibrosis pathway is also highly conserved. In addition, since almost all the signaling pathways described here are also involved in embryonic development, as well as carcinogenesis, these results demonstrate the delicate balance and context-dependent nature of the fibrotic processes (80). Of course, given the highly mechanically active nature of skeletal muscle, it is also likely that many (if not all) of these signaling pathways and morphogenic transitions will themselves be mechanosensitive.

Cells Involved in Skeletal Muscle Fibrosis

While collagen deposition by myofibroblasts is involved in skeletal muscle fibrosis models, the cellular source of myofibroblasts in various conditions is less clear (Fig. 8). As noted previously in this series, certain cell types transdifferentiate into myofibroblasts (see Fig. 2 of Ref. 131). The detailed fate-mapping and lineage-tracing experiments in studies of

other tissues have not been done in studies of skeletal muscle. Numerous cell sources have been suggested for myofibroblasts in muscle, including muscle-derived stem cells (72), resident fibroblasts (120), and, as observed in injured arteries and skin wounds (25), fibrocytes (56), perivascular cells (20), and nerve-associated cells (57).

Recently, skeletal muscle fibrosis and adipogenesis have been linked by the discovery of an adult muscle progenitor cell population: fibroadipogenic progenitors (FAPs) (63, 125). These cells, which are uniquely identified by their expression of the PDGF receptor- α (PDGFR α), do not differentiate into muscle fibers *in vivo* or *in vitro*. Transplantation experiments revealed that the differentiation of FAP cells into fat or connective tissue was strongly affected by the local environment (Fig. 9). The precise molecular cues for this differentiation are not known. The central role in fibrosis of cells that express PDGFR α was vividly demonstrated by experiments in which

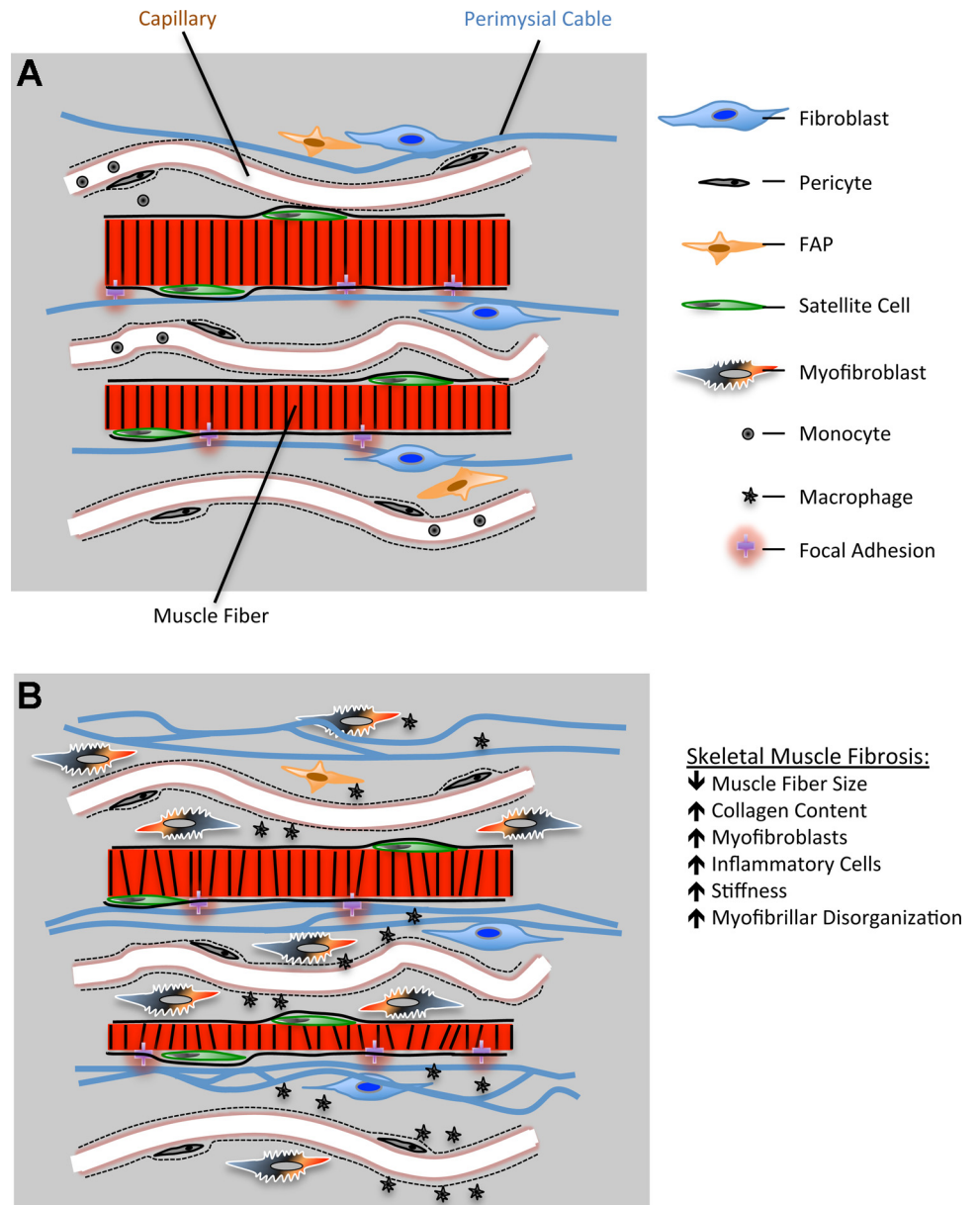


Fig. 8. *A*: schematic illustration of the location of cells involved in skeletal muscle fibrosis. Skeletal muscle contains numerous sources of mononuclear cells that can ultimately become activated fibroblasts (myofibroblasts; multicolored). These sources include inflammatory cells (black), resident fibroblasts (blue), fibroadipogenic progenitor (FAP) cells (orange), and pericytes (black). There is no evidence that satellite cells (green) become myofibroblasts. *B*: after an insult that results in fibrosis (cf. Table 2), muscle tissue is characterized by muscle fiber atrophy, increased collagen content, presence of myofibroblasts and inflammatory cells, increased muscle mechanical stiffness, and myofibrillar disorganization that presents primarily as disrupted muscle cell z disks.

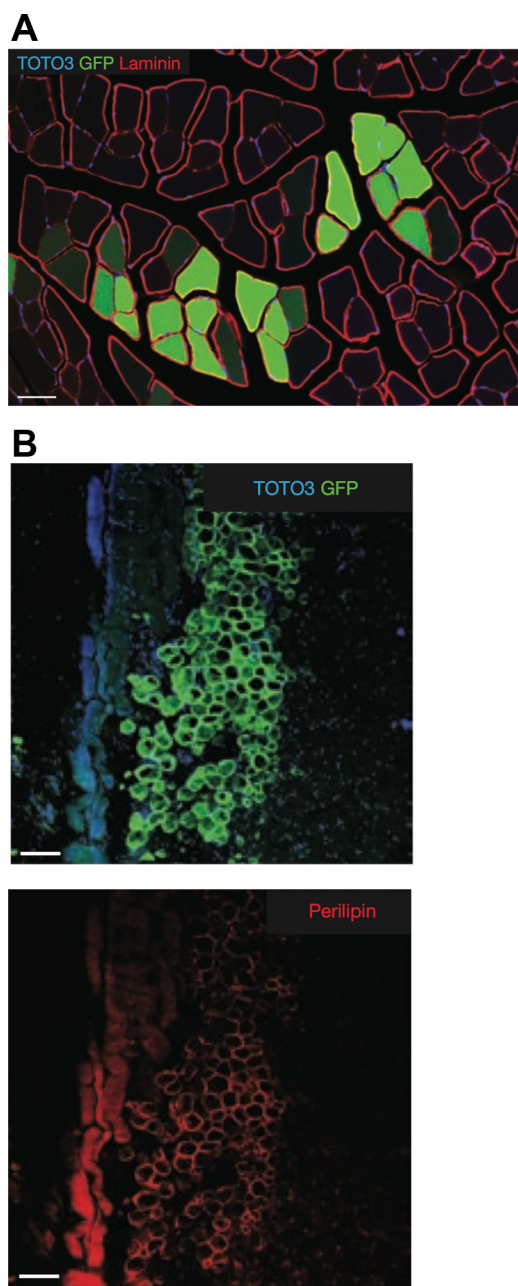


Fig. 9. Two different progenitor cells differentiate into either muscle or fat. *A*: myogenic precursor cells (identified as $CD34^+/Sca1^-$; see Ref. 63) that express green fluorescent protein (GFP) were injected into muscle and engrafted to existing muscle fibers that were damaged along the needle track. Red outline of the fiber indicates laminin immunostaining. *B*: adipogenic and fibrogenic precursor cells (identified as $PDG\alpha^+/CD34^+/Sca1^+$; see Ref. 63) that express GFP were injected subcutaneously and differentiated into adipocytes, as evidenced by the positive perlipin stain. Scale bars, 50 μm . (Images from Ref. 63.)

chronic $PDGFR\alpha$ activation resulted in widespread organ fibrosis (95), but the extent to which FAPs play a role in skeletal muscle fibrosis is not clear. In fact, the role of all these cell types in muscle fibrosis is not clear. Perhaps conditions with different causes recruit different populations of cells.

With regard to skeletal muscle as a mechanotransducer of the environment, it is interesting to suggest that the differentiation process relates in part to the biomechanical microenvi-

ronment in muscle tissue. Perhaps altered ECM mechanical properties help direct the fate of resident progenitor cells. Of note are the experiments of Engler et al. (30), who found that mesenchymal stem cells differentiate into neural, muscular, or bony cells, depending on the biomechanical properties of the substrate, thus demonstrating the strong effect of local mechanics on progenitor cells. Mesenchymal stem cells grown on substrates that approximate the elasticity of muscle (~ 10 kPa) express a wide array of myogenic genes, such as MyoD, Myf5, myogenin, and Pax7. This expression was largely inhibited by the contractile inhibitor blebbistatin. A substrate mechanical property that is more bonelike (~ 100 kPa) favors expression of osteogenic genes, including Bmp4, Sox9, and Col1A. It would be interesting to titrate elasticity to more closely approximate ECM properties and test if the expression pattern is more fibrogenic (Table 1). We recently found that the ECM of muscle from children with cerebral palsy is compromised in its mechanical properties relative to the ECM of children with normal development (76). It is intriguing to conjecture that part of the defect in these muscles that leads to contracture directly results from changes in ECM properties that are secondary to the neural insult. Clearly, more experiments are needed to sort out the cellular source, cellular mechanisms, and functional significance of fibrosis in normal skeletal muscle as well as the numerous diseases and model systems of fibrosis.

A recent, novel finding is that activated fibroblasts can arise from the endothelial cells that line the skeletal muscle microvasculature (24). Dulauroy et al. (24) focused on the metalloprotease ADAM12 (a disintegrin and metalloprotease 12), since it is expressed in the tissues of human diseases that involve fibrosis of liver, muscle, and skin. In view of this expression of ADAM12, they used genetic ablation of ADAM12, along with inducible fate mapping, to show that the majority of myofibroblasts arose from the muscle's perivascular cells that transiently expressed ADAM12 during development. This finding complements evidence that subsets of pericytes are the major sources of myofibroblasts and scar tissue in kidney (59) and spinal cord (47). If ADAM12 is selectively overexpressed in muscle, one finds increased muscle fibrosis and suppressed skeletal muscle regeneration (64). Since ADAM12 is rapidly induced by $TGF\beta$ (24, 112), this would represent a potential positive-feedback loop for the fibrotic response.

Conclusions

The physiological basis of skeletal muscle fibrosis is more poorly understood than processes that occur in other organs, such as kidney (15), liver (131), and heart (41). The most obviously measurable effects of skeletal muscle fibrosis are the increase in ECM collagen and increased mechanical stiffness of muscle fiber bundles. An explicit quantitative relationship between collagen content and passive mechanical properties of skeletal muscle is not available due to limited understanding of the structural organization of ECM collagen.

Skeletal muscle fibrosis can represent a debilitating clinical problem that may even require surgical intervention for correction. Nonsurgical therapeutic approaches involve targeting the classic $TGF\beta$ pathway, as well as other systems involved in muscle ECM homeostasis, such as Cthrc1 expression (113), Wnt signaling (11), and perivascular cell turnover (24). As in other fibrotic tissues, myofibroblasts deposit collagen, and

these cells may derive from transdifferentiation of resident fibroblasts, FAPs, pericytes, and mesoangioblasts. Given this large number of cell sources and myriad underlying biological processes, future studies will require prodigious use of fate-mapping and molecular and cellular techniques to create powerful rescue and prevention strategies that inhibit or even reverse skeletal muscle fibrosis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.L.L. and S.R.W. prepared the figures, R.L.L. and S.R.W. drafted the manuscript, R.L.L. and S.R.W. edited and revised the manuscript, R.L.L. and S.R.W. approved the final version of the manuscript.

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