Tenotomy immobilization as a model to investigate skeletal muscle fibrosis (with emphasis on Secreted frizzled-related protein 2)

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Akpulat U, Onbaşlar İ, Kocaefe YÇ. Tenotomy immobilization as a model to investigate skeletal muscle fibrosis (with emphasis on Secreted frizzled-related protein 2). Physiol Genomics 48: 397–408, 2016. First published April 25, 2016; doi:10.1152/physiolgenomics.00010.2016.—The pathological endpoint of congenital and senile myopathies is chronic muscle degeneration characterized by the atrophy of contractile elements, accompanied by fibrosis and fatty infiltration of the interstitium. Tenotomy is the release of preload that causes abrupt shortening of the muscle and models atrophy and fibrosis without prominent inflammatory response. Fibrosis in the skeletal muscle is known to be triggered by transforming growth factor (TGF)-β, which is activated by inflammatory events. As these were lacking, tenotomy provided an opportunity to investigate transcriptional events on a background without inflammation. An unbiased look at the transcriptome of tenotomy-immobilized soleus muscle revealed that the majority of the transcriptional changes took place in the first 4 wk. Regarding atrophy, proteasomal and lysosomal pathways were actively involved in accompanying cathepsins and calpains in the breakdown of the macro-molecular contractile machinery. The transcriptome provided clear-cut evidence for the upregulation of collagens and several extracellular matrix components that define fibrotic remodeling of the skeletal muscle architecture as well as activation of the fibro-adipogenic precursors. Concomitantly, Sfrp2, a Wnt antagonist as well as a procollagen processor, accompanied fibrosis in skeletal muscle with an expression that was stringently confined to the slow-twitch fibers. An interpreted mechanistic scenario construed the kinetic events initiated through the abnormal shortening of the muscle fibers as enough to trigger the resident latent TGF-β in the extracellular matrix, leading to the activation of fibroadipogenic precursors. As in the heart, Sfrp2 shows itself to be a therapeutic target for the prevention of irreversible fibrosis in degenerative skeletal muscle conditions.

Tenotomy; immobilization; fibrosis; muscle atrophy; Sfrp2

CHRONIC MUSCLE DEGENERATION is the pathological endpoint of progressive degenerative conditions such as inherited defects of structural muscle proteins and senile deterioration of skeletal muscle. The hallmarks of degeneration are muscle atrophy accompanied by fibrosis and fatty infiltration of the interstitium. These three cardinal features also dominate the pathological picture of Duchenne muscular dystrophy (DMD), one of the most common single gene disorders of childhood. Animal models are widely in use to investigate the regeneration process and the testing of therapeutic applications such as gene and cell therapies in skeletal muscle, but the isogenic models of human dystrophies do not reflect the same pathological picture. The murine model (mdx mouse) exhibits intensive regeneration with the exception of diaphragm, and the deterioration observed in the mdx is much milder than in DMD boys (45). On the contrary, Golden Retriever muscle dystrophy, the canine model, displays extensive fibrosis resulting in severe contractures and deformities (22, 40). The fibrosis component of the muscular dystrophies is gaining further attention in line with accelerating clinical applications aiming to correct genetic defects. Once a proven remedy is available, the next challenge would be to understand and reverse fibrosis, because it is a restrictive factor limiting the efficacy of currently proposed therapeutic interventions (53). Thus, better models are needed to observe and understand the molecular events, signals, pathways, and mediators that shape the architectural changes of degenerating muscle.

Eliminating the physiological preload of the muscle by tendon release or tenotomy causes abnormal shortening, which triggers atrophy and fibrosis without any inflammatory response. Tenotomy provokes a rapid myofiber atrophy reflected at the pathological level by central core lesions (1) without inducing new fiber formation (16). The tenomized muscle accurately represents the chronic tissue architecture changes that occur in dystrophies such as fibrosis. High-throughput gene expression profiling provides an opportunity to observe global gene expression changes in pathological conditions that exhibit differentiation as well as structural and tissue architecture changes. Thus, we conducted a gene expression profiling study to observe the transcriptional events that shape the fibrotic and deteriorating muscle in response to tenotomy.

A number of systemic pathogenic mediators that act on initiation and progression of fibrosis have been previously identified. These global factors act on the initiation and progression of fibrosis, regardless of the anatomic localization. Transforming growth factor (TGF)-β (54) connective tissue growth factor (42, 51), and platelet-derived growth factor (PDGF) (6) are such common fibrotic mediators that also act on skeletal muscle. Myostatin, however, was identified to be specific to the skeletal muscle, directly activating intrinsic fibroblasts in addition to regulating the muscle mass (26, 55).

One recently identified mediator of myocardial fibrosis is the Secreted frizzled-related protein 2 (Sfrp2), which is a classical inhibitor of Wnt signaling (30). The fibrotic effect of Sfrp2 in myocardial remodeling is devoid of action on the Wnt pathway. Novel but controversial roles are attributed to Sfrp2, either as an inhibitor of Bmp1 (11) or an enhancer of procollagenase (18). Here we describe, for the first time, the upregulation of Sfrp2 concomitantly with the early fibrotic events that initiate fibrosis in skeletal muscle. Transcription data from open access databases also support the influence of Sfrp2 in muscle degeneration.

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MATERIALS AND METHODS

Animals and surgical procedures. The tenotomy and denervation were performed on 3 mo old male Sprague-Dawley rats weighing 250 g (± 40 g). All animal procedures were performed in accordance with the institution-approved protocol (H.U.2005/40-8 for tenotomy and H.U.2007/11-1 for denervation), under strict biological containment. Following 90/10 mg/kg ip of ketamine-xylazine anesthesia and appropriate disinfection, tenotomy of the Achilles tendon was performed as follows: the tendon was exposed by a longitudinal incision on the right ankle skin, and an ~4 mm distal section was removed. The proximal end was folded back and superficially sutured to the gastrocnemius muscle to delay reattachment during wound healing. The animals were randomly assigned to time-course groups of 1, 2, 3, 4, 6, 16, and 22 wk, and each group consisted of at least five animals. Selected time-matched extremity muscles of the left hindlimb were designated as control samples. At the predetermined time following the intervention, soleus muscles from both the right and left extremities were resected under general anesthesia and the animals were reanesthetized by cervical dislocation. The soleus muscle was dissected out and sectioned into three identical transverse segments, where the midbelly was freshly processed for RNA extraction. The proximal and distal segments were fresh-frozen accordingly for cryosectioning and protein extraction procedures. Denervation of the sciatic nerve was conducted by standard procedure. Following anesthesia, the sciatic nerve of the right side was exposed, and an 8 mm segment of the nerve was resected from the midhigh level. The harvesting and processing of the denervated soleus and gastrocnemius muscles were performed in the same way as the tenotomy muscle specimens.

Immunostaining and histochemistry. The explanted muscle specimens were immediately dipped in tissue embedding solution and freshly frozen in isopentane cooled with liquid nitrogen. Samples were stored at ~80°C until 6 μm thick cryosectioning was performed using a Leica 1900 cryotome. Tissue sections were investigated using standard hematoxylin-and-eosin (h.e.) staining for the tissue morphology, and Sirius red staining was employed to document the collagen deposition. Cytochrome oxidase (COX) staining was applied to normal and denervated samples as described previously (35). Standard fluorescent immunostaining was conducted to document the localization of Sfrp2 (Sigma HPA002652, rabbit polyclonal, 1:300 dilution) as well as myosin heavy chain (MHC) (slow isoform, Novocastra, mouse monoclonal, NCL-MHCs, 1:40 dilution) and desmin (Sigma DEU-10, mouse monoclonal, 1:40). The samples were incubated with fluorescent labeled appropriate secondary antibodies. DAPI staining was applied to visualize the nuclei, and images were captured on a Leica DMIL microscope equipped with a Leica DFC 320 camera (Leica).

RNA extraction. We directly transferred 50–75 mg of resected muscle to a 2 ml bead-beater tube containing ceramic beads and 1 ml of tissue-lysing solution (Fastprep pro green, MP biochemicals). The disrupted tissues were kept at ~80°C and simultaneous RNA extraction was performed on all samples upon manufacturer’s recommendations. RNA quality and quantity were assessed and documented by denaturing agarose gel electrophoresis and optic density absorbance measurements, respectively.

Gene-chip experiments. We reverse-transcribed 10 μg of total RNA into cDNA by means of Superscript II reverse transcriptase, using an Affymetrix one-cycle cDNA synthesis kit. In vitro transcription into cRNA, fragmentation, hybridization onto gene chips and detection of the signal intensities were pursued according to manufacturer recommendations, using standard Affymetrix protocol and reagents. RAE_230 (2.0) rat gene expression chips were used for the assessment of global gene expression of the selected samples.

Analysis of the microarray data. The workflow of the microarray data analysis was as follows: Affymetrix cel files were subjected to RMAExpress 0.5 release (26 Feb 2007) (13). The normalized gene-chip expression data was then loaded onto BRB Array Tools version 4.2.1 (January 2012). Initially, an unbiased visualization of the data was conducted by clustering the samples, using centered correlation and supervised linkage based on all genes by using principal component analysis. This approach yielded the identification of the outliers as well as the samples that clustered significantly. The genes that were differentially expressed were identified by random-variance t-test. This class comparison analysis was conducted on the two clustered groups of samples, excluding the outliers, with a significance threshold cutoff P value <0.001, restricting the maximal false discovery proportion to 1%. This gene list was further filtered by a fold-change cutoff of 2. Further clustering and visual analysis were conducted using this generated list of significantly altered genes. Following annotation of the genes, the fold-change values of the probe-sets corresponding to the same gene were averaged. Furthermore, functional clustering was conducted using the gene ontology classification tools of DAVID 6.7 (http://david.abcc. nci.nih.gov/). The relative fold change of expression values, as well as the random-variance t-test significance values for the above-mentioned generated gene list and all other relevant gene lists are presented as additional supplementary tables. The Affymetrix cel. file raw data as well as the analyzed data were deposited to the Gene Expression Omnibus (GEO) database and are available for access (GEO dataset GSE73510).

Protein and mRNA expression analysis. Protein extraction from muscle tissue specimens was performed as described previously (19). The total protein concentrations of the cellular extracts from time-course samples were determined by bicinchoninic acid (BCA) Protein Assay (Pierce, Rockford, IL), and 80 μg of total protein was loaded onto 12% SDS-PAGE gel. Following the transfer of proteins onto the nitrocellulose membrane (Bio-Rad, Hercules, CA), equal loading was verified by Ponceau-S staining, and the success of the transfer was confirmed by Coomassie blue gel stain. The membrane was incubated consecutively with rabbit polyclonal anti Sfrp2 (Protein-tech, 12189-1-AP) and mouse monoclonal anti-α-tubulin (Sigma, T6074). Generation of the signal was accomplished by appropriate horseradish peroxidase-conjugated secondary antibodies (Invitrogen, G21234, G21040) using a chemiluminescence detection system (SuperSignal West Femto maximum sensitivity substrate - Thermo Scientific). Sfrp2 gene expression levels and other relevant genes were validated by fluorometric quantitative PCR using SYBRGreen technique. Appropriate primers from exon junctions were used for the selective amplification of cDNAs on a Rotorgene 3000 (Corbett Life Science) PCR instrument. The specificity of the amplicon was validated by gel electrophoresis. Quantitative PCR studies were performed on biological replicates of five samples for each time point. The quantitative expression results were normalized to the expression of beta actin and were then further normalized to the average expression of the control samples. The average expression values were presented as a fold change relative to the control samples.

Isolation of mononucleated cells. Extracted soleus muscles were freshly minced by scalpsels on ice and digested with 1% collagenase B and dispase-II solution in PBS buffer for 20 min with gentle agitation. Digested samples were sequentially filtered through 70 and 40 μm mesh filters and subjected to Ficoll gradient centrifugation. Isolated mononuclear cells from the muscle tissue were enumerated and lysed in Trizol solution (Invitrogen) and stored at ~80°C until RNA extraction.

Quantitation of mtDNA. DNA extraction was conducted on fresh frozen tissue samples, control samples (n = 5) and the first 4 wk tenotomy samples (n = 3 for each week). Slices of fresh frozen tissues were subjected to proteinase K digestion, and DNA extraction was performed according to standard phenol-chloroform extraction procedure. Comparative quantitative PCR was performed using SYBRgreen technique in separate triplicate amplifications.

1 The online version of this article contains supplemental material.
using gene-specific primers targeting mtDNA CytB gene normalized to the 18S ribosomal RNA gene. Comparative mtDNA quantitation was achieved by normalizing the average of control samples to the average of tenotomized muscle replicates. The values are provided as fold changes.

RESULTS

Consequences of tenotomy on muscle. Tenotomy is the release of physiological preload of muscle, which induces rapid gross changes such as shrinkage of the vertical size and increase in midbelly diameter. Tenotomy provokes endomysial fibrosis as well as myofibrillar atrophy (16). After the resection of the Achilles tendon, the gross investigation of the tenotomized muscles did not reveal any necrosis or inflammatory events in either gastrocnemius or soleus muscles. The mass ratios of the tenotomized (right) and control (left) soleus were measured before freezing and compared. A reduction in muscle mass was evident from the first week (0.9 ± 0.1 g for tenotomized right, 1.2 ± 0.1 g for control left). This initial drop of mass was not progressive throughout the observation period and the ratio remained stable at 0.75 (± 0.1) until the sixth week. The fibrotic organization of the skin and a tendon-like sclerotic regeneration were observed progressively in the anatomic localization of the resected Achilles tendon. This was the most evident from the fourth week onward, indicating a partial recovery without religation. The gross morphology as well as the muscle mass of the 16 and 22 wk tenotomized muscles were identical to the contralateral controls.

Microscopic examination of the h.e. sections revealed central core lesions in atrophic fibers as well as rounding and loss of the polygonal fiber morphology starting from the first week. The morphology of the myonuclei also revealed prominent enlargement and rounding. As indicated in the gross findings above, these progressive observations confined to the myofibers exhibited a gradual reversal starting from the sixth week. However, centrally located nuclei were not observed in any of the fibers, indicative of a lack of regenerating myofibers (Fig. 1). The perimysium, as well as the endomysial space between the fibers, displayed a prominent widening beginning in the first week. As the endomysial expansion deduced fibrosis in the skeletal muscle, Sirius red staining was employed to specifically demonstrate increasing collagen deposition (27). This approach revealed the presence of dense collagen bundles in the endomysial space, confirming interstitial fibrosis (Fig. 1). The observed expansion of the endomysial space was not progressive, but rather stabilized following the second week. The microscopy of the 16 and 22 wk tenotomized muscle revealed fiber morphology that was similar to the controls but contained persistent deposits of endomysial collagen. The histological studies did not suggest any evidence for the infiltration of mononuclear cells, increase in vascularization or any fatty infiltration.

Gene expression profiling. The whole gene expression profile was initially used to establish an unbiased clustering of the samples. This approach revealed that the control samples clustered together with samples from the 6, 16, and 22 wk tenotomized muscles. These late time-point samples, as well as the controls, were clearly separated from the tenotomy samples of the first 4 wk. Figure 2 shows the visual distribution of the samples by principal component analysis (Fig. 2A) and clustering dendrogram (Fig. 2B). This distribution demonstrated that the majority of the transcriptional events shaping the fibrotic remodeling of the muscle architecture took place in the first 4 wk. In light of this finding, a supervised class comparison analysis was conducted to identify the differentially altered profile. A random-variance $t$-test revealed 2,412 signifi-

![Fig. 1. Typical representative microscopy images of the time-course histological investigations are provided. Hematoxylin and eosin (h.e.) staining reveals enlargement of both endomysial and perimysial space (top). Central core lesions (marked with arrowheads) are prominently visible in some of the fibers in the first 4 wk. The enlargement and rounding of the myonuclei was most evident in the 1st week. Prominent collagen deposition was demonstrated by Sirius red staining (bottom). Despite the recovery of the fiber morphology in 6 wk samples, endomysial and perimysial collagen deposits were persistent. Size bar represents 50 μm.](http://physiolgenomics.physiology.org/)
The sole TGF-β signaling pathway is a key regulator of fibrotic progression, was not detectable, and the expression of TGF-β1 expression was upregulated up to fourfold in both control and tenotomized muscles (Fig. 3B). To identify the spatial source of Sfrp2 expression, we employed two approaches. First, the Sfrp2 immunostaining on the tenotomized soleus muscles exhibited expression confined to only some of the fibers, sparing others. Costaining with a monoclonal antibody specific to the slow-twitch isoform of MHC revealed that Sfrp2 expression was exclusively confined to slow-twitch fibers (Fig. 3C). Sfrp2 immunoreactivity was not detected in control samples where tenotomy was not applied. An alternative approach was conducted to confirm the spatial expression pattern of Sfrp2. The mononucleated cells of 1 wk tenotomized soleus muscle (any other cells besides myofibers) were isolated. Western blot results revealed no detectable Sfrp2 expression in the mononuclear cell compartment, confirming the spatial localization of Sfrp2 solely to the slow-twitch myofibers (Fig. 3B). Sfrp2 expression was also investigated in soleus and gastrocnemius muscles following denervation. As in the tenotomy, Sfrp2 expression was immunolocalized to Type 1, slow-twitch fibers in both soleus and gastrocnemius muscles (Fig. 4A). Denervation also triggered Sfrp2 expression. In soleus muscle, this induction reached up to sixfold in 2 wk. In gastrocnemius muscle, denervation caused a limited upregulation of twofold, while tenotomy caused a modest upregulation, limited to less than threefold (Fig. 4B).

The expressions of other selected transcripts relevant to fibrosis were also validated by quantitative real-time PCR. A quantifiable TGF-β1 amplification could not be achieved in RT-PCR studies, confirming the microarray results. The basal expression of TGF-β1 in both control and tenotomized muscles did not reveal any upregulation. TGF-β2 expression did not exhibit any significant variance among controls or tenotomy samples, but TGF-β3 expression was upregulated up to fourfold in the tenotomy samples of the first 4 wk. Similarly, fourfold upregulation of Bmp1 expression validated the transcriptome studies (data not shown).

Quantitative mtDNA assessment and COX staining. The gene expression data clearly depicted that the majority of the downregulated genes exclusively contained mitochondrial transcripts (structural proteins as well as enzymes). To demonstrate the impairment of mitochondrial biogenesis in tenotomized muscle, we assessed the mtDNA copy number. Quantitative PCR analysis revealed up to 80% depletion of the...
mitochondrial DNA in tenotomy samples from the first 4 wk (Fig. 5). The impairment of mitochondrial function was further verified by COX staining, which revealed diffuse loss of mitochondrial function in tenotomized muscle. Furthermore, COX staining representing mitochondrial function was abruptly localized to the subsarcolemmal space and the central core lesions (Fig. 5, bottom).

DISCUSSION

Chronic muscle degeneration is a complex process involving a multitude of events affecting different tissue compartments. In dystrophic muscle, cycles of necrosis, regeneration, and atrophy are the dominating events confined to muscle fibers. Concomitantly, the progressive interstitial remodeling involves fibrosis and fatty infiltration in the dystrophic muscle. These events are further complicated by a prominent inflammatory response triggered by the necrotic fibers. Today, understanding fibrosis in skeletal muscle is of particular interest for several reasons, primarily due to the fact that fibrotic remodeling affects the niche of satellite cells, thus disrupting the required environment for activation, migration, and fusion, ultimately rendering them inutile for regeneration (5). Second, fibrosis is the main obstacle in the delivery of any therapeutic remedy to the myofibers (53). Third, even if the primary genetic defects underlying muscle dystrophies might be corrected, fibrosis, the kinetically restrictive component of the dystrophic muscle, cannot be reversed (14). Thus, attempts to understand the molecular mediators of fibrosis are attracting more attention for the benefit of clinical interventions. Release of the preload by tenotomy induces clear-cut fibrosis in the muscle without disrupting the integrity of sarcolemma, providing a unique model to study the events specifically leading to fibrosis. This model is distinct from other models of disuse atrophy such as hindlimb or tail suspension, where a certain amount of atrophy is observed but no fibrosis is induced (39, 43). On the other hand, denervation causes dramatic atrophy of the myofibers and remodeling of the perimysium, but in contrast to myopathies, endomysial fibrosis is limited (21). Tenotomy-induced fibrosis is devoid of prominent myofiber necrosis. Thus, the secondary inflammatory response is very limited, confined to the sites of segmental necrosis. However, ECM remodeling and fibrosis in muscle dystrophies are initiated by rounds of

Table 1. Functional annotation clustering of the upregulated and downregulated genes

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Only the clusters with an enrichment score >10 are shown.

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extensive necrosis and regeneration accompanied by prominent inflammation.

**Atrophy.** Focusing on myofibers, microscopic examination revealed prominent central core lesions suggesting the breakdown of contractile elements through proteasomal degradation (1). As previously reported, these lesions were the most evident in the first- and second-week samples and declined until the fourth week (4). There was no detectable regenerative myofiber response to tenotomy, indicated by the absence of myofibers with small diameters and central nuclei and further confirmed by a negative immunostaining for neonatal myosin heavy chain (not shown). Furthermore, transcriptome data were not indicative of any regenerative response, lacking upregulation in myogenic regulatory factors. Following functional recovery, polygonal myofiber morphology was restored after 6 wk, but fibrosis of the endomysial space remained persistent.

Muscle atrophy is characterized by the breakdown of myofibers, by either proteasomal or lysosomal degradation. Published gene and protein expression profiling studies show strong diversity in terms of dominating protein degradation pathways, depending on the investigated models or disease conditions (28). Proteasomal degradation of contractile elements is regulated through Foxo1 and Foxo3, which in turn regulate ubiquitin-protein ligases Trim63/MuRF1, Atrogin1/MAFbx, and NEDD4. Among these, NEDD4 had been previously identified as the cardinal ubiquitin ligase responsible for unloading-related muscle atrophy (20). Concordantly, we observe that tenotomy induces a fourfold upregulation of NEDD4, which also satisfies nonparametric statistical significance (*P > 0.05). Western blot analysis confirmed the upregulation of Sfrp2 at the protein level (B). Typical protein samples from tenotomized muscles in the 1st, 2nd, and 3rd wk (W) reveal remarkable increase in specific immunoreactivity compared with the control samples (C1, C2). The protein extract of the mononuclear cells isolated from 1 wk tenotomized muscle did not reveal any specific immunoreactivity, demonstrating the lack of expression in this tissue compartment (MC). Immunostaining for Sfrp2 (in red) and myosin heavy chain(s) [MHC(s) in green] antibodies reveals that Sfrp2 expression was strongly confined to the slow-twitch fibers in tenotomized muscle (typical immunostaining results from 1 wk tenotomized soleus muscle are provided in C). Size bar represents 100 μm top and 20 μm bottom.
atrophy (7- and 3-fold, respectively). These subunits were previously reported to act upon denervation-induced atrophy and correspond to E2 and E3 proteasomal subunits (37).

The second major pathway in muscle atrophy is lysosomal degradation, also known as the autophagy pathway. Foxo3 transcription factor also regulates the autophagy pathway independently of the proteasomal degradation. This regulation is mediated through its downstream effector, Bnip3, which encompasses a dual action: first, Bnip3 and Bnip3l are both responsible for the Foxo3-mediated induction of autophagosome formation (28). Second, Bnip3l also mediates mitophagy in atrophying muscle; twofold upregulation of Bnip3l, the Foxo3-mediated atrogene, is supportive of the involvement of mitochondrial remodeling in tenotomy-induced atrophy (36). The twofold upregulation of Bnip3l supports the contribution of this major breakdown pathway in tenotomized muscle. The degradation of the skeletal muscle in autophagosomes is mediated through the lysosomal hydrolases. Among these hydrolases, cathepsin L (which lies downstream of Bnip3) was previously described as being involved in disuse muscle atrophy in hindlimb suspension (41) and denervation (37). In tenotomy-induced atrophy, lysosomal cathepsins B and H (but not cathepsin L) were upregulated threefold, supporting an induction in lysosomal enzymes. Previously reported transcriptome data of muscle biopsies from myopathy patients further support the involvement of these two cathepsins in atrophy.

It has been documented that both of these abovementioned systems may be sufficient for the breakdown of the monomeric contractile elements but are inadequate for the degradation of the large protein complexes of the sarcomere (17). Experimental evidence suggests that the breakdown of the sarcomere structure is coordinately primed by two mechanisms: caspases 1 and 3 (23) and the calcium-dependent proteinases, calpains, (8, 44) are suggested to be responsible for this priming act. In tenotomized muscle, caspases 1 and 3 are significantly upregulated 3.7- and 4.5-fold, respectively. Likewise, the expression of calpain 1, 5, and 6 is upregulated 4-, 5-, and 9-fold, respectively.

The major mediators triggering skeletal muscle atrophy are ubiquitously present and activated through posttranslational modifications (such as Foxo3). Thus, the transcription profiles of these key mediators may not correlate with their respective activities (50). So far, however, the gene expression data presented here show that the downstream components of the two major degradation pathways, ubiquitin-proteasome and lysosomal/autophagy, were simultaneously actively involved in tenotomy-induced atrophy, along with other accessory pathways. A schematic diagram showing the upregulated (in red) or downregulated (in green) genes in tenotomy-mediated atrophy is presented in Fig. 6. The sole pathway that did not reveal any involvement was the NF-κB pathway, which is cardinally activated in catabolic states such as sepsis or renal failure and
requires the presence of TNF-α in circulation (23). Similarly, Hunter et al. (12) have previously shown that the canonical NF-κB pathway was not activated in hindlimb unloading.

**Fibrosis.** Tenotomy induced a rapid upregulation in all ECM components as well as collagens from the first week of immobilization in rat soleus muscle. As far as the transcriptome analysis reveals, this upregulation consists of muscle-specific collagens (I, III, IV, V) as well as structural ECM components such as laminin beta1, fibronectin, fibulin, and perlecan. Jarvinen et al. (14) have previously investigated the padded tape-mediated immobilization model in the lower extremity of rats and described the upregulation of collagen III in response to immobilization. Here we observed that collagen type I was the dominantly upregulated subtype (>20-fold for Col1a1 and 10-fold for Col1a2) along with others listed in Supplementary Table S2. In almost every tissue fibrosis can be deduced de facto by an increase in collagen type 1 along with pathologic conditions. Several studies have pinpointed the role of infiltrating M1 macrophages in priming the fibrotic events through the release of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and macrophage inflammatory protein 2 (46). Mononuclear infiltration in the tenotomy model was ruled out by histological investigation of tissue sections and gene expression data. The lack of necrotic fibers or direct tissue damage is a likely explanation. Meticulous examination of the gene expression data revealed approximately two- to threefold upregulation in some transcripts that are unique to monocytes and macrophages (such as Ada, Ccl6, Ccl2). This observation is further in line with the upregulation of a number of acute-phase proteins, such as adipsin, and lipopolysaccharide-bind-

![Fig. 5. mtDNA copy numbers were assessed throughout the 4 wk tenotomized soleus muscle (n = 5 for each time point). The relative mtDNA copy numbers were normalized to the average of the controls (top). Cytochrome oxidase staining verified diminishing oxidative phosphorylation activity (bottom).](image)

The known inflammatory events and mediators that interplay in dystrophic muscle were absent in tenotomy. The cardinal initiator of fibrosis in dystrophic skeletal muscle is TGF-β. TGF-β1 is the major and global subtype responsible for fibrosis (54). Quantitative investigation of TGF-β isoforms was not conclusive. TGF-β1 expression was undetectable in both tenotomy and control samples, and the modestly expressed TGF-β2 was not altered. TGF-β3 expression was upregulated 3.5-fold, which in itself was not enough to explain the fibrotic events. Considering the lack of inflammatory cells and mediators that activate TGFs, other pathways and mediators of tissue remodeling and fibrosis need to be considered. Two potential scenarios are drawn to explain fibrosis and fibroblastic cells in tenotomy. First, activated muscle precursors can be misdifferentiated into myofibroblasts (7). Second, the heterogeneous nonsatellite cell population residing in skeletal muscle can be activated to proliferate and acquire fibroblast phenotypes. Current research efforts are just beginning to understand and characterize these mesenchymal progenitor cells that define the latter scenario. Two recently identified markers for the myofibroblastic cells are Tcf4 and Pdgfra (33, 34, 48). The transcriptome data presented here depict 3.7- and 2.9-fold upregulation of these marker transcripts, respectively. However, for a fibrogenic differentiation, this cell population needs to be activated through the TGF-β pathway as well (25, 49). In tenotomy-induced fibrosis, TGF-β isoforms were not observed to be deregulated at the transcriptional level. However, the activation of the “latent TGF-β” residing in the ECM is capable of priming the observed fibrotic events and merits further elucidation. Nevertheless, the observed upregulation of LTBP 2 and 3 (4.5- and 3.6-fold, respectively), linking adapters of TGF-β to ECM is also favorable for enhanced TGF-β signaling in tenotomy-induced fibrosis. In addition to these, it has been previously reported that TGF-β1 and myostatin reciprocally induce the expression of one another as well as the fibrotic events in regenerating muscle (55). It is highly likely that this vicious cycle of reciprocal induction is counteracted by follistatin, the physiological antagonist of myostatin in tenotomy. The probable source of the observed 6.25-fold increase in follistatin levels is likely to be the activated fibro/adipogenic progenitors themselves (32).

The answer to the activation of the latent TGF-β may lie in the mechanical dynamics of the tenotomy. The preload release immediately caused an instant and abnormal shortening as well as a prominent increase in muscle diameter. These immediate physical changes are likely to provoke two events in the structure: first, the reflection of the increase in muscle diameter would provoke an extensive mechanical stretch, creating a tension on the membrane and connective tissue, as well as the ECM. Such mechanical stress was previously shown to activate latent TGF-β as well as the downstream signaling in vascular tissue, intestine, liver, and lungs (10, 15, 31, 38). Second, the abnormal shrinkage of the sarcomere structure in
Fig. 6. A diagram representing major pathways and components interplaying in skeletal muscle atrophy and hypertrophy is presented. The genes exhibiting upregulation are designated in red, and those exhibiting downregulation are represented in green. Major components of the proteasomal degradation pathway are upregulated as well as a number of caspases and calpains.

Fig. 7. Hypothetical interpretation of the events that trigger transforming growth factor (TGF)-β activation after tenotomy is presented in the diagram. Tenotomy causes a sudden and abrupt shortening of the muscle height, which causes increase in the diameter, thus causing excessive tension in the sarcolemma. This mechanical tension is expected to trigger the activation of latent TGF-β in the extracellular space as well as the disruption of sarcoplasmic reticulum and intracellular calcium release. Respectively, these events are proposed to trigger fibrosis in the extracellular space and breakdown of sarcomeric components via activation of proteases.
tenotomy is known to disrupt the sarcoplasmic reticulum (3, 4), causing local calcium release (1). A schematic interpretation of this pathomechanical scenario is presented in Fig. 7. Further examination is required to determine whether these events (primarily the former) are capable of triggering the activation of the latent TGF-β resident in the ECM directly or through the activation of Bmp1.

Sfrp2. One recently identified modulator of ECM components and fibrotic events in cardiac muscle is Sfrp2, which functions as a dose-dependent procollagenase enhancer (11, 18). Following tenotomy, Sfrp2 expression was observed to be upregulated more than 30-fold and strongly confined to the slow-twitch myofibers undergoing atrophy, not present in the control specimens or mononuclear cells. Investigation of open-access gene expression data of various muscle pathology samples (2) shows up to threefold Sfrp2 upregulation in dystrophic muscle known to exhibit fibrosis (data not shown). The sequence and the speed of the events precipitated by tenotomy are expected to be different from chronic progressive myopathies. Moreover, two points need to be considered for the correct interpretation of Sfrp2 expression. First, the source of expression is solely the slow-twitch fibers, and second, based on the exclusively slow-twitch background of the soleus, the Sfrp2 upregulation is expected to be observed more clearly. To support this postulate, we conducted a comparative evaluation of Sfrp2 expression of gastrocnemius and soleus muscles following denervation. The upregulation was detectable in gastrocnemius but was much more limited than that of soleus (Fig. 4B), mirroring highly limited fibrosis in denervation (21). Interestingly, Sfrp2 expression was also strikingly upregulated during early myogenesis as well as injury repair and confined to the slow-twitch fibers until maturation (24). While the role of Sfrp2 in muscle development is still obscure, if one considers the attributed biochemical role as an enhancer of procollagenase C activity of Bmp1, it is highly likely that this induction potentiates the profibrogenic accumulation of collagens released to the extracellular space. Thus, this may be linked to the architectural modelling of the ECM during development. However, under stress conditions (such as tenotomy), Sfrp2 expression from slow-twitch fibers also accompanies fibrosis. In the light of these supportive findings, significance of Sfrp2 expression in chronic degenerative conditions of skeletal muscle requires further elucidation. If exogenously administered Sfrp2 (supraphysiological levels) or interventions through blocking antibodies also inhibit fibrosis in the muscle as they do in the heart (11, 29), this may pinpoint Sfrp2 as a potential drug target for fibrosis in dystrophies as well.

Sfrp2 is a known Wnt inhibitor. While Wnt signaling is important for myogenesis (47), the role in activation of satellite cells and injury regeneration is controversial (52). The transcriptome of the tenomized soleus did not show alterations in any of the genes related to Wnt signaling. It has been previously shown that Sfrp2 is upregulated during injury regeneration (52) and exerts a differentiation inhibitory action on fibroblasts (9). Thus, Sfrp2 may act to divert activated myoblasts to a myofibroblastic fate, contributing to fibrosis (7). However, in the case of tenotomy, the myofiber integrity was well preserved, and the lack of evidence for satellite cell activation renders this scenario to be highly unlikely.

Conclusions

The transcriptome analysis presented in this study reveals several unique features of tenotomy-immobilized muscle. Tenotomy is one appropriate model in which to observe and study the cardinal structural changes as well as the molecular events, mediators, and pathways that deteriorate skeletal muscle. Novel therapy avenues toward the cure of myopathies, including interventions of genetic correction, are rapidly progressing. Prevention of fibrosis is important as it irreversibly affects muscle function as well as the delivery and success of therapeutics. Thus, molecular mediators defining the pathogenesis of fibrosis are gaining further importance. While Tgf-β is one cardinal mediator at the junction of a multitude of pathways, the lack of availability of research tools for animal models (such as an active TGF-β antibody for rodents) is a significant obstacle limiting further investigation. The consequences of tenotomy on the maintenance of mitochondria reported here require further investigation at the functional level. Our future efforts will be directed toward the elucidation of molecular events that trigger fibrosis in skeletal muscle. Nevertheless, as in the heart, it is essential to evaluate if Sfrp2 harbors any potential in skeletal muscle to counteract fibrosis.

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REFERENCES


