MUSCULOSKELETAL PATHOLOGY

***KLF10* Gene Expression Modulates Fibrosis in Dystrophic Skeletal Muscle**

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Dystrophic skeletal muscle is characterized by fibrotic accumulation of extracellular matrix components that compromise muscle structure, function, and capacity for regeneration. Tissue fibrosis is often initiated and sustained through transforming growth factor- β (TGF- β) signaling, and Krüppel-like factor 10 (*KLF10*) is an immediate early gene that is transcriptionally activated in response to TGF- β signaling. It encodes a transcriptional regulator that mediates the effects of TGF- β signaling in a variety of cell types. This report presents results of investigation of the effects of loss of *KLF10* gene expression in wild-type and dystrophic (mdx) skeletal muscle. On the basis of RT-PCR, Western blot, and histological analyses of mouse tibialis anterior and diaphragm muscles, collagen type I (*Col1a1*) and fibronectin gene expression and protein deposition were increased in *KLF10*^{-/-} mice, contributing to increased fibrosis. *KLF10*^{-/-} mice displayed increased expression of genes encoding SMAD2, SMAD3, and SMAD7, particularly in diaphragm muscle. *SMAD4* gene expression was unchanged. Expression of the extracellular matrix remodeling genes, *MMP2* and *TIMP1*, was also increased in *KLF10*-deficient mouse muscle. Histological analyses and assays of hydroxyproline content indicated that the loss of *KLF10* increased fibrosis. Dystrophic *KLF10*-null mice also had reduced grip strength. The effects of loss of *KLF10* gene expression were most pronounced in dystrophic diaphragm muscle, suggesting that *KLF10* moderates the fibrotic effects of TGF- β signaling in chronically damaged regenerating muscle. (*Am J Pathol* 2018, 188: 1263–1275; <https://doi.org/10.1016/j.ajpath.2018.01.014>)

The murine X-linked muscular dystrophy (mdx) mouse is an animal model of human Duchenne muscular dystrophy. Both disease states are characterized by complete absence of the dystrophin protein, a component of the dystrophin-glycoprotein complex that mediates cytoskeleton connectivity to the muscle fiber extracellular matrix (ECM). Loss of dystrophin destabilizes muscle cell integrity during contraction, leading to muscle fiber necrosis.¹ Appendicular musculature in mdx mice exhibits varying amounts of muscle fiber degeneration and regeneration, depending on muscle fiber type and age. Fast twitch muscle fibers, such as those that comprise the tibialis anterior (TA) muscle, are preferentially, but not exclusively, affected.² Extensive muscle degeneration begins at approximately 4 weeks of age, with compensatory regeneration peaking at approximately 6 weeks. By week 10, degeneration has subsided to low persistent levels, and regeneration of the acute degeneration event is complete.³ Interestingly, unlike appendicular musculature, the mdx diaphragm muscle is characterized by chronic progressive

degeneration with accompanying functional reductions in strength and twitch speed.⁴ The histopathology of the mdx diaphragm is, therefore, more closely analogous to the progressive muscle wasting in Duchenne muscular dystrophy patients.

Dystrophic skeletal muscle is also characterized by progressive fibrosis, an excessive accumulation of ECM components. In skeletal muscle, fibrosis primarily involves expansion of the endomysium and perimysium. Both collagen type I and fibronectin are principle components of the ECM in fibrotic muscle tissue.^{5,6} Within fibrotic skeletal muscle, muscle fiber size is often reduced, contributing to loss of muscle cell volume and mass. However, the nature of the mechanistic relationship between increased ECM accumulation and reduced muscle fiber size is not well understood and may well be dependent on the underlying cause of fibrosis

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and persistence of the causative factors (eg, chronic degenerative disease versus repeated trauma). Regardless of the causative factors, muscle fibrosis is typically associated with compromised muscle function, including reduced strength and increased dynamic stiffness. The contribution of individual components of fibrotic ECM, such as collagen and fibronectin, to compromised muscle function remains unclear and is not always readily correlated with all measures of altered function.⁷ The regenerative capacity of fibrotic skeletal muscle is also compromised.⁸ Although mdx muscle fibers have increased numbers of satellite cells and increased proliferative response to mitogenic factors, such as fibroblast growth factor 2,⁹ net muscle regeneration in the mdx diaphragm and Duchenne muscular dystrophy muscle is outpaced by muscle fiber necrosis and fibrosis. Ultimately, fibrosis is a major impediment to skeletal muscle recovery of normal structure and function in the dystrophic state.^{6,10,11}

Transforming growth factor- β (TGF- β) is central to regulation of the regenerative response to skeletal muscle injury and disease as well as the cellular and molecular mechanisms that lead to fibrosis. TGF- β signaling, through membrane-associated TGF- β type I and type II receptors, stimulates fibroblasts to increase production of collagen and fibronectin.^{12,13} TGF- β activity also regulates expression of ECM remodeling proteins, such as matrix metalloproteinases (MMPs), tissue inhibitors of MMPs (TIMPs), and plasminogen activator inhibitor type 1 (PAI-1).¹⁴ In addition, TGF- β activity controls the regenerative capacity of muscle satellite cells. TGF- β represses myogenic cell differentiation by interference with myogenin-mediated gene expression.^{15,16} Levels of TGF- β are increased in mdx mouse diaphragm and gastrocnemius muscles.^{12,17} The role of TGF- β in the mdx phenotype is demonstrated by studies that reduce TGF- β activity. Expression of a dominant negative TGF- β receptor (TGF- β R) and administration of a TGF- β neutralizing antibody both reduced mdx muscle fibrosis.^{18,19} Conversely, muscle-specific TGF- β 1 overexpression in transgenic mice yielded increased fibrosis and muscular atrophy.²⁰

Expression of TGF- β —inducible early gene 1 [alias Krüppel-like factor 10 (*KLF10*)] is induced soon after cell exposure to TGF- β . *KLF10* gene expression is induced as early as 30 minutes after TGF- β treatment and reaches a maximum within 2 hours of induction. *KLF10* protein levels reach a maximum shortly thereafter (2 to 3 hours).²¹ *KLF10* is expressed in human fetal osteoblasts, mesangial cells, fibroblasts, T cells, hippocampal neurons, PC12 cells, and mouse cardiomyocytes.^{22–28} *KLF10* is a C₂H₂ zinc finger transcription factor that functions as a transcriptional regulator of TGF- β responsive genes. The KLF family of transcriptional regulators contains members that can function as transcriptional activators or repressors by binding to GC-rich *cis* elements in transcriptional regulatory regions. Although *KLF10* mediates TGF- β signaling via positive transcriptional regulation, it also can function as a transcriptional repressor. For example, in avian skeletal muscle cells, *KLF10* interacts with a GC element in the upstream regulatory region of the fibroblast growth factor receptor 1

(*FGFR1*) gene, repressing its expression. This effect on *FGFR1* gene expression generally correlates with the altered proliferation and differentiation of C₂C₁₂ cells overexpressing *KLF10*.^{29,30} *KLF10* also mediates TGF- β signaling through transcriptional regulation of genes encoding SMAD isoforms. *KLF10* represses *SMAD7* gene expression, but conversely activates *SMAD2* gene expression.^{31,32} This altered SMAD signaling, particularly SMAD2/3 signaling, has subsequently been linked to muscle fibrosis and loss of muscle regenerative capacity, characteristic of the dystrophic phenotype.^{33,34}

KLF10-deficient mice display a variety of defects. They display an osteopenic phenotype with significantly weakened femurs, reduced cortical bone thickness, and reduced vertebral bone volume.³⁵ This is likely because of a decrease in the total number of osteoblasts and corresponding reduction in the rate of bone formation, especially in female mice.³⁶ Fast and slow twitch muscle fibers from *KLF10*-deficient mice have altered mechanical properties.³⁷ They also display connective tissue defects in tendon ultrastructure after injury, with delayed expression of TGF- β and delayed healing.³⁸ X-ray microdiffraction experiments revealed that tail tendon fibers of *KLF10*^{-/-} mice are disorganized, with a total decrease in the amount of collagen in the tendons.³⁹ Interestingly, *KLF10*^{-/-} mice display cardiac defects, including hypertrophic cardiomyopathy with increased fibrosis, indicating a possible role for *KLF10* in modulating fibrosis.⁴⁰ Therefore, the loss of *KLF10* affects production and deposition of ECM components, apparently with tissue-specific outcomes.

This study analyzed the effects of *KLF10* gene deletion on expression of genes in the TGF- β signaling pathway with a focus on those genes related to fibrosis in skeletal muscle tissue. The study also analyzed the histopathology of TA and diaphragm muscles from *KLF10*-deficient mice compared with wild-type mice and from *KLF10*-deficient mdx mice compared with mdx mice. The functional effect of *KLF10* loss was assessed by grip strength measurements.

Materials and Methods

Animals

KLF10^{-/-} mice were generated, as previously described,⁴¹ and obtained from Dr. Thomas C. Spelsberg (Mayo Clinic, Rochester, MN). Briefly, exons 1 and 2 as well as 2.3 kb of 5' flanking region were replaced with the neomycin resistance gene in a targeting vector, which was then injected into C57BL/6 blastocysts. *KLF10*^{-/-} mice in a congenic background were obtained after breeding against the C57BL/6 wild-type mouse background for at least eight generations.³⁶ *KLF10*^{-/-} mice were bred against the mdx mouse genetic background (C57BL) for eight generations to yield *KLF10*^{-/-}/mdx mice.

Real-Time Quantitative RT-PCR

RNA was obtained from 1-year-old mouse diaphragm and TA muscles using RNA-Stat 60 reagent, according to the

manufacturer's instructions (Tel-Test Inc, Friendswood, TX). cDNA synthesis from RNA samples was primed with oligo-dT and random hexamers. Amplification was performed in triplicate using real-time quantitative RT-PCR SYBR Green master mix, including ROX passive reference dye (Applied Biosystems, Foster City, CA). *GAPDH* mRNA amplification was used as a reference for normalization of amplification variability across samples. Expression levels of each gene were calculated by determination of $\Delta\Delta C_T$. The oligonucleotides used for amplification are listed in Table 1.

Western Blot Analysis

Protein from 1-year-old mouse diaphragm and TA muscles was extracted by glass grinder homogenization in ice-cold protein extraction buffer, pH 7.4 (50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA, 10 mmol/L NaF, 10 mmol/L sodium orthovanadate, and 20 mmol/L sodium pyrophosphate). Homogenized extracts were set on ice for 10 minutes and then centrifuged for 10 minutes at $16,000 \times g$ in an Eppendorf centrifuge at 4°C. Supernatants were collected and stored at -80°C. Protein samples were denatured by addition of $2 \times$ Laemmli's buffer, followed by incubation at 95°C for 5 minutes. Samples were electrophoresed in 7.5% polyacrylamide gels and transferred to nitrocellulose membrane. Blots were incubated in blocking solution [5% nonfat dry milk, 0.05% Tween-20 in phosphate-buffered saline (PBS)] overnight at 4°C. Blots were then incubated in primary antibody, diluted in blocking solution, for 1 hour at room temperature. The following antibodies were used at the indicated dilutions: KLF10 at 1:100 (AB85970; Abcam, Cambridge, UK), fibronectin at 1:100 (C-20; Santa Cruz Biotechnology, Dallas, TX), embryonic myosin heavy chain (EMyHC) at 1:10 (CRL2039; ATCC, Manassas, VA),

SMAD2 at 1:200 (511300; Life Technologies, Carlsbad, CA), SMAD3 at 1:200 (511500; Life Technologies), SMAD4 at 1:200 (AB40759; Abcam), SMAD7 at 1:200 (216428; Abcam), TGF- β R at 1:200 (AB31013; Abcam), and α -actin at 1:5000 (A2172; Sigma, St. Louis, MO). Blots were washed five times with PBS plus 0.05% Tween-20 and then incubated in secondary antibody (Santa Cruz Biotechnology) diluted 1:2000 in blocking solution for 1 hour at room temperature. Blots were washed as before. Protein bands were visualized using the ECL Chemiluminescence Detection Kit (GE Healthcare, Little Chalfont, UK), according to the manufacturer's instructions. Protein band intensities were quantitated using ImageJ software version 1.46 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>) and normalized against corresponding actin protein band intensities.

Histological Analysis

Diaphragm and TA muscles from 1-year-old mice were frozen in OCT Compound (Sakura Finetek, Torrance, CA) and cryosectioned (10 μ m thick). Sections were obtained throughout the midportion of each muscle with at least 100- μ m separation between sections. Six sections were obtained for each muscle. Some sections were stained for collagen using Masson trichrome stain, according to the manufacturer's instructions (Sigma). Other serial sections were immunostained at room temperature, as follows. Sections were fixed in 4% formaldehyde in PBS for 10 minutes and then washed in PBS twice for 5 minutes each. For immunostaining with the phosphorylated (phospho)-SMAD2/3 antibody, sections were then immersed in ice-cold methanol for 10 minutes, followed by a wash in PBS for 5 minutes at room temperature. Sections were then incubated in blocking solution (5% horse serum, 2% bovine serum albumin in PBS) for 1 hour. Most primary antibodies, diluted in blocking solution, were applied for 1 hour at room temperature. The phospho-SMAD2/3 antibody was applied overnight at 4°C. The following antibodies were diluted as indicated: anti-laminin at 1:500 (AB11575; Abcam), anti-fibronectin at 1:100 (SC8422; Santa Cruz Biotechnology), anti-EMyHC at 1:5, and phospho-SMAD2/3 at 1:200 (8828; Cell Signaling Technology, Danvers, MA). Sections were washed in PBS, and the following secondary antibodies, diluted in blocking solution as indicated, were applied to the serial sections for 1 hour: fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:100) to detect anti-laminin antibody, Texas red-conjugated anti-mouse IgG (1:100) to detect anti-fibronectin and anti-EMyHC antibodies, and Texas red-conjugated anti-rabbit IgG to detect the phospho-SMAD2/3 antibody. Sections were stained with DAPI for 10 minutes and then washed with PBS, as before. Images of sections were collected using a Nikon Eclipse 80i epifluorescence microscope (Nikon, Tokyo, Japan). For determination of muscle fiber cross-sectional areas, microscopic fields at $\times 20$ magnification were randomly selected, and the area within each muscle fiber basement membrane, as shown by laminin immunostaining, was outlined

Table 1 Primers Used for RT-PCR

Gene	Oligonucleotides
<i>GAPDH</i>	F: 5'-GACCCCTTCATGACCTCAACTAC-3' R: 5'-CACGGAGGCCATGCCAGYGAG-3'
<i>KLF10</i>	F: 5'-CTGACTGCATCAGCGCCACTCTACTGGCC-3' R: 5'-CATCTGGCAGATGACAGGCAGGGGTGGC-3'
<i>Col1a1</i>	F: 5'-GAAGAACTGGACTGTCCCAACC-3' R: 5'-ACCTTGAATCCAGTAGCACCG-3'
Fibronectin	F: 5'-GACGCATCACCTGTACCTCCAG-3' R: 5'-CATCAACGATGCACTGGTCTCG-3'
<i>MMP2</i>	F: 5'-CGCGTAAAGTATGGGAACGCTG-3' R: 5'-GTAGTTGGTTGTGGTTCGCACAC-3'
<i>TIMP1</i>	F: 5'-AATGCCGCAGATATCCGGTACG-3' R: 5'-TGGCAGGCAAGCAAAGTGACGG-3'
<i>SERPINE1</i>	F: 5'-CGGATGCCATCTTTGTCCAGCG-3' R: 5'-AGATGTTGGTGAGGGCGGAGAG-3'
<i>SMAD7</i>	F: 5'-CCAACTGCAGGCTGTCCAGATG-3' R: 5'-CACAAAGCTGATCTGCACGGTG-3'

F, forward; R, reverse.

and quantified by Nikon Elements software version 3.22. All muscle fibers within a field were measured. Six fields per muscle section were analyzed. For determination of the percentage of muscle tissue composed of muscle fibers, microscopic fields were randomly selected, and individual muscle areas were summed to obtain the collective area of all muscle fibers within the microscopic field. The area of the field of view was quantified by Nikon Elements software. The sum of the muscle fiber areas was then divided by the total tissue area within the field of view. Six microscopic fields per muscle section were analyzed. Six sections of each muscle, isolated from six different mice, were analyzed.

Hydroxyproline Assay

Hydroxyproline content was measured by an assay based on previously developed assays.^{42,43} Muscle tissue was carefully trimmed of connective tissue, and wet weights were measured. The tissue was then hydrolyzed in 2 N NaOH in Eppendorf tubes placed in boiling water for 30 minutes. After cooling to room temperature, 10 to 50 μ L aliquots of the sample in a total volume of 50 μ L of 2 N NaOH were added to 450 μ L of chloramine T solution [1.46 g chloramine T trihydrate (01,779; Thermo Fisher Scientific, Waltham, MA) in 5.2 mL H₂O, followed by addition of 13 mL of buffer solution (250 mmol/L citric acid, 1.2% acetic acid, 880 mmol/L sodium citrate, and 850 mmol/L NaOH, pH 6.0), and then 7.8 mL of 2-methoxyethanol with gentle warming to fully dissolve the chloramine T]. The mixture was incubated at room temperature for 25 minutes. Ehrlich's Reagent [5 g p-dimethylaminobenzaldehyde (D-71; Fisher) in 22.2 mL of absolute ethanol and 11.1 mL concentrated sulfuric acid] was freshly prepared for each assay, and 500 μ L of the reagent was added to the reaction, which was then mixed and incubated at 65°C for 20 minutes. Absorbance of the solution at 560 nm was determined using a Genesys 10S spectrophotometer (Thermo Fisher Scientific). Hydroxyproline content was calculated using a standard curve of 0 to 20 μ g purified hydroxyproline (H54409; Sigma) and normalized against the total volume of the hydrolyzed sample and the muscle tissue wet weight.

Grip Strength

Mouse forelimb grip strength was measured using a grip strength meter equipped with a pull bar (Columbus Instruments, Columbus, OH) and set at peak tension mode. Five male and five female mice of each genotype were assessed. Each mouse was tested five times with a 3-minute rest period between each repetition.

Statistical Analysis

Data were analyzed using one-way analysis of variance. For real-time quantitative RT-PCR and Western blot analyses, gene expression data from the tibialis anterior of *KLF10*^{-/-},

mdx, and *KLF10*^{-/-}/*mdx* mice were compared with data from the tibialis anterior of wild-type mice. Similarly, gene expression data from the diaphragm of the experimental genotypes were compared with data from the wild-type diaphragm control. Tibialis anterior and diaphragm gene expression from *KLF10*^{-/-}/*mdx* mice were also compared with gene expression from the respective wild-type control muscles. Data from experiments that quantified phospho-SMAD, collagen content, muscle fiber area, percentage area muscle fiber, and grip strength were similarly analyzed using analysis of variance. Before analysis of variance analyses, data groups were verified to have equal variance, with a confidence of 95% ($\alpha = 0.05$) using a two-tailed F-test. For pairwise comparisons, Welch's *t*-test was applied, followed by Bonferroni correction method using SPSS software version 24 (IBM, Armonk, NY). Statistical significance was established with $P < 0.05$, unless otherwise indicated.

Results

Gene Expression Analysis

Initial characterization of the effects of *KLF10* gene deficiency in skeletal muscle focused on genes indicative of fibrosis [eg, collagen type 1 (*Colla1*) and fibronectin] and genes associated with remodeling of extracellular matrix [eg, *MMP2*, *TIMP1*, and *Pai1* (official name *SERPINE1*)]. Characterization was performed by analyses of gene expression using real-time quantitative RT-PCR (Figure 1). RNAs were isolated from 1-year-old diaphragm and TA muscles from wild-type, *KLF10*^{-/-}, *mdx*, and *KLF10*^{-/-}/*mdx* mice. Analysis was performed using mice at 1 year of age to allow for induction of fibrosis over time under normal conditions of activity. With regard to *KLF10* gene expression, modest levels of RNA were detected in wild-type diaphragm and TA muscles, as revealed by gel electrophoresis of amplified cDNA product. Both diaphragm and TA muscles from *mdx* mice had significantly higher levels of *KLF10* gene expression. *KLF10* gene expression in *mdx* diaphragm and TA muscles was increased approximately 2.3- and 0.5-fold, respectively, compared with wild-type muscle. As expected, *KLF10* gene expression was not detected in *KLF10*^{-/-} or *KLF10*^{-/-}/*mdx* diaphragm or TA muscles.

Analyses of *Colla1* and fibronectin gene expression revealed increased levels in *KLF10*^{-/-} mice. *Colla1* gene expression was consistently increased in diaphragm muscle compared with TA muscle in all four mouse genotypes tested. Within diaphragm muscles, no significant differences in *Colla1* gene expression were detected in *KLF10*^{-/-} or *mdx* diaphragm and TA muscles compared with wild-type muscles. However, both diaphragm and TA muscles from *KLF10*^{-/-}/*mdx* mice had increased levels of *Colla1* gene expression relative to wild-type, *KLF10*^{-/-}, and *mdx* mouse muscles. Unlike *Colla1*, fibronectin gene expression in wild-type and *KLF10*^{-/-} diaphragm muscle was not

Figure 1 Gene expression analysis of *KLF10*-deficient wild-type and mdx mice. RNAs were isolated from tibialis anterior (T) and diaphragm (D) muscles of 1-year-old wild-type (WT), *KLF10*^{-/-}, mdx, and *KLF10*^{-/-}/mdx mice. RT-PCR was performed, as described in *Materials and Methods*, for the following genes: *KLF10*, collagen type I (*Col1a1*), fibronectin (FN), matrix metalloproteinase 2 (*MMP2*), tissue inhibitor of MMPs 1 (*TIMP1*), plasminogen activator inhibitor type 1 (*PAI1*), and *SMAD7*. *GAPDH* was used as a normalizing control. Data are expressed as means ± SEM relative gene expression levels. *n* = 6. **P* < 0.05 experimental tibialis anterior versus wild-type control; †*P* < 0.05 experimental diaphragm versus wild-type control; ‡*P* < 0.05 *KLF10*^{-/-}/mdx versus mdx tibialis anterior; §*P* < 0.05 *KLF10*^{-/-}/mdx versus mdx diaphragm.

increased relative to TA muscle. However, both mdx and *KLF10*^{-/-}/mdx diaphragm and TA muscles had increased levels of fibronectin gene expression relative to wild-type and *KLF10*^{-/-} mouse muscles. Moreover, fibronectin gene expression in *KLF10*^{-/-}/mdx diaphragm muscle was significantly increased compared with mdx diaphragm muscle. In total, these results indicate that loss of *KLF10* gene expression increased *Col1a1* and fibronectin gene expression, particularly in the diaphragm muscle of mdx mice.

Expression of genes associated with extracellular matrix remodeling was also analyzed. *MMP2* gene expression was significantly, but modestly, increased in both diaphragm and TA muscles from *KLF10*^{-/-} mice compared with wild-type controls. The mdx diaphragm displayed increased expression relative to wild-type diaphragm, and both diaphragm

and TA muscles from *KLF10*^{-/-}/mdx mice had increased *MMP2* gene expression relative to wild-type muscles. Also, loss of *KLF10* gene expression in mdx TA muscle (*KLF10*^{-/-}/mdx) increased *MMP2* gene expression. *TIMP1* gene expression did not significantly vary between diaphragm and TA muscles across each of the genotypes tested. Similarly, *PAI-1* gene expression did not significantly change.

Previous research has shown that *KLF10* can repress *SMAD7* gene expression, which normally down-regulates TGF-β-induced cell signaling.³² In *KLF10*^{-/-} and *KLF10*^{-/-}/mdx mice, *SMAD7* gene expression was increased in both diaphragm and TA muscles compared with wild-type and mdx mice, respectively. These results indicate that expression of profibrotic *Col1a1* and fibronectin genes occurs despite increased *SMAD7* gene expression.

Western blot analysis was performed on whole-cell extracts of diaphragm and TA muscles from 1-year-old wild-type, *KLF10*^{-/-}, mdx, and *KLF10*^{-/-}/mdx mice (Figure 2). KLF10 protein was detected in mdx diaphragm extracts only. Fibronectin protein was detected in all cell extracts, with an increased abundance in extracts from mdx and *KLF10*^{-/-}/mdx diaphragm muscle extracts. Furthermore, fibronectin protein content was increased in *KLF10*^{-/-}/mdx versus mdx diaphragm muscle extract. EMyHC was detected in diaphragm muscle from both mdx and *KLF10*^{-/-}/mdx mice. It was not detected in any extracts of TA muscle. TGF- β 1 protein was detected in all cell extracts at similar levels.

Differences in protein content for SMAD2, SMAD3, SMAD4, and SMAD7 were observed. SMAD2 protein was detected in each cell extract, except those extracts from wild-type and mdx diaphragm. In contrast, SMAD3 protein was only detected in appreciable amounts in cell extracts from

KLF10^{-/-}/mdx mice. Both diaphragm and TA muscles from *KLF10*^{-/-}/mdx mice contained SMAD3 protein. SMAD4 protein was detected in all cell extracts, and diaphragm muscle from *KLF10*^{-/-}, mdx, and *KLF10*^{-/-}/mdx mice contained more detectable SMAD4 protein (approximately 2.5-fold) compared with wild-type diaphragm and all TA muscle. Last, and in agreement with RT-PCR data, SMAD7 protein was detected in diaphragm muscle from both *KLF10*^{-/-} and *KLF10*^{-/-}/mdx mice. These results demonstrate differences between diaphragm and TA muscles with respect to genotype-specific detection of SMAD2 and SMAD7. The results also demonstrate non-muscle-specific differences in SMAD3 protein content, with detectable levels in both muscle types restricted to the *KLF10*^{-/-}/mdx genotype only.

To further investigate the effect of loss of *KLF10* on SMAD2/3 function in wild-type and dystrophic diaphragm and TA muscles, muscle sections were immunostained with a

Figure 2 Detection and quantitation of proteins by Western blot analyses. **A:** For Western blots, protein extracts from 1-year-old tibialis anterior (TA) and diaphragm muscles were obtained from wild-type (WT), *KLF10*^{-/-} (K), mdx (M), and *KLF10*^{-/-}/mdx (KM) mice. Proteins were resolved and detected using protein-specific antibodies (Materials and Methods). α -Actin served as a loading control. The blot shown is representative of analyses of three mice of each genotype. **B:** Protein bands were quantitated using ImageJ software version 1.46 and normalized to the corresponding α -actin protein bands to compare protein abundance with muscle fiber protein content. There were significant differences in the means of protein content between muscles of experimental genotypes and the respective wild-type controls. Data are expressed as means $\pm$ SEM relative gene expression levels. $n = 3$ (B). * $P < 0.05$, ** $P < 0.01$ versus wild-type. EMyHC, embryonic myosin heavy chain; FN, fibronectin; TGF- β R, transforming growth factor- β receptor.

Figure 3 Detection and quantitation of phosphorylated (phospho)-SMAD2/3 (pSMAD2/3)—positive nuclei. **A:** Cryosections of tibialis anterior (TA) and diaphragm muscles from 1-year-old wild-type, *KLF10*^{-/-}, mdx, and *KLF10*^{-/-}/mdx mice were immunostained with a phospho-SMAD2/3—specific antibody. Nuclei were stained with DAPI. A serial section was immunostained with a laminin-specific antibody. Images were added to each other using Photoshop CS6 software version 13.0.6 (Adobe Systems, San Jose, CA). Phospho-SMAD2/3 and laminin antibodies were detected using a Texas red— and fluorescein isothiocyanate—conjugated secondary antibody, respectively. **Arrows** indicate phospho-SMAD2/3—positive nuclei. **B:** The number of phospho-SMAD2/3—positive nuclei relative to all nuclei per microscopic field was determined. Six fields were selected at random from each section, and six sections per muscle were analyzed. Muscle samples were collected from six mice of each genotype. Means of percentage pSMAD—positive nuclei of each genotype were compared with wild-type (WT), *KLF10*^{-/-}, and mdx. ***P* < 0.01 versus WT; ††*P* < 0.01 versus *KLF10*^{-/-}; ‡‡*P* < 0.01 versus mdx. Scale bar = 100 μm (A).

phospho-SMAD2/3—specific antibody to detect active nuclear localized SMAD2/3 (Figure 3A). Phospho-SMAD2/3 was not detected in sections of wild-type diaphragm and TA muscles. Similarly, nuclear localized SMAD2/3 was not detected in sections of mdx diaphragm and TA muscles. As anticipated, centrally located nuclei in both mdx muscle sections were readily apparent, indicative of regenerated muscle fibers.⁴⁴ Phospho-SMAD2/3 was not detected in sections of TA muscle from *KLF10*^{-/-} and *KLF10*^{-/-}/mdx mice. However, phospho-SMAD2/3 was present in sections of diaphragm muscle from these mice. Phospho-SMAD2/3 immunostaining colocalized with DAPI staining of nuclei. The relative number of phospho-SMAD2/3—positive nuclei was determined for each muscle of each genotype (Figure 3B). Significantly greater numbers of phospho-SMAD2/3—positive nuclei were observed in diaphragm muscle from *KLF10*^{-/-} versus wild-type and from *KLF10*^{-/-}/mdx versus mdx mice.

Assessment of Fibrosis and EMyHC Gene Expression

Cryosections of wild-type, *KLF10*^{-/-}, mdx, and *KLF10*^{-/-}/mdx diaphragm and TA muscles were stained for the fibrotic markers, collagen and fibronectin. Collagen was detected with Masson trichrome stain (Figure 4). Wild-type diaphragm and TA muscles showed no appreciable accumulation of collagen. Similarly, the TA muscle of *KLF10*^{-/-} muscle did not stain for collagen. Minor

accumulations of collagen were noted in *KLF10*^{-/-} diaphragm muscle sections. TA muscle sections from mdx mice also displayed minor collagen accumulation. More pronounced collagen deposition was observed in mdx diaphragm muscle and in *KLF10*^{-/-}/mdx TA muscle. Most noticeably, significant collagen deposition was observed in diaphragm muscle sections from *KLF10*^{-/-}/mdx mice.

Analysis of hydroxyproline content was performed to provide additional quantitative assessment of fibrosis, as defined by collagen content, in TA and diaphragm muscles of each genotype (Figure 5). Muscles were carefully trimmed of visible connective tissue outside the muscle epimysium, weighed, and hydrolyzed, as described in *Materials and Methods*. The amount of hydroxyproline relative to muscle wet weight in TA muscles from mice across all four genotypes was not significantly different. With regard to diaphragm muscle, there was no significant difference in hydroxyproline content between wild-type and *KLF10*^{-/-} mice. Yet, both mdx and *KLF10*^{-/-}/mdx mice had significantly more hydroxyproline compared with wild-type mice. More important, diaphragm muscle from *KLF10*^{-/-}/mdx mice had approximately 48% more hydroxyproline than mdx diaphragm muscle.

Similar accumulation of fibronectin was observed in sections using an anti-fibronectin antibody (Figure 6). Fibronectin is a normal constituent of muscle fiber basal lamina and was detected in lamina surrounding each fiber. However, significant amounts of fibronectin were detected

Figure 4 Histological analysis of fibrosis. Cryosections of tibialis anterior (TA) and diaphragm muscles from 1-year-old wild-type, *KLF10*^{-/-}, mdx, and *KLF10*^{-/-}/mdx mice were stained with Masson trichrome stain to detect collagen (blue). Sections were counterstained with eosin (red) to visualize muscle fibers. The images are representative of analyses of six mice of each genotype. Scale bar = 50 μ m.

in interstitial spaces of diaphragm muscle from mdx and *KLF10*^{-/-}/mdx mice, with the most noticeable accumulation in the latter. These results, coupled with quantitation of fibronectin protein content via Western blot analysis, indicate extensive fibrosis in *KLF10*^{-/-}/mdx mice, with the most pronounced fibrosis detected in the diaphragm muscle.

EMyHC gene expression in TA and diaphragm muscle cross sections from 1-year-old mice was assessed by immunodetection using an EMyHC-specific monoclonal antibody (Figure 7). EMyHC was consistently absent from TA muscle, regardless of the genotype tested. EMyHC was also absent from diaphragm muscle of wild-type and *KLF10*^{-/-} mice. However, EMyHC was detected in diaphragm muscle from both mdx and *KLF10*^{-/-}/mdx mice. The percentage of muscle fibers expressing EMyHC was 1.8 ± 0.6 and 2.3 ± 0.8 in mdx and *KLF10*^{-/-}/mdx diaphragm muscles, respectively. These values are not significantly different.

Muscle Morphometry

To determine whether the absence of the *KLF10* gene affected muscle fiber size and the relative contribution of contractile muscle fibers to overall muscle size, TA and diaphragm muscle cross sections from each mouse genotype were

immunostained with an anti-laminin antibody to detect muscle fiber basal lamina (Figure 7). Muscle fiber cross-sectional areas within the basal lamina were measured as described in *Materials and Methods* (Figure 8). The average cross-sectional area of TA muscle fibers in *KLF10*^{-/-} mice did not vary significantly from the average cross-sectional area of wild-type TA muscle fibers. Similarly, the average cross-sectional areas of *KLF10*^{-/-} diaphragm muscle fibers were not significantly different from wild-type muscle fibers. However, the average areas of both mdx and *KLF10*^{-/-}/mdx muscle fibers were significantly reduced compared with wild-type and *KLF10*^{-/-} muscle fibers. Significant reductions in muscle fiber areas were evident in both TA and diaphragm muscles. The mdx and *KLF10*^{-/-}/mdx muscle fibers in both muscles showed similar reductions in average cross-sectional areas of approximately 35% relative to wild-type and *KLF10*^{-/-} muscle fibers, respectively.

Analysis of the relative proportion of contractile muscle fiber area to total muscle cross-sectional area revealed relative reductions in contractile tissue area in each of the experimental genotypes compared with wild-type control muscle (Figure 8). TA muscle from *KLF10*^{-/-}/mdx demonstrated a modest, but significant, reduction in total muscle fiber area compared with wild-type TA muscle. With regard to diaphragm muscle, the

Figure 5 Analysis of collagen content. The amounts of hydroxyproline were measured in tibialis anterior (gray bars) and diaphragm (black bars) muscles from 1-year-old wild-type (WT), *KLF10*^{-/-}, mdx, and *KLF10*^{-/-}/mdx mice. Hydroxyproline content, as determined by comparison to a standard curve of known amounts of hydroxyproline, was normalized to muscle wet weight. Means of collagen content of each genotype were compared with wild-type, *KLF10*^{-/-}, and mdx mice. Data are expressed as means $\pm$ SEM. $n = 6$. ** $P < 0.01$ versus WT; †† $P < 0.01$ versus *KLF10*^{-/-}; ‡‡ $P < 0.01$ versus mdx.

Figure 6 Immunolocalization of fibronectin protein. Cryosections of tibialis anterior (TA) and diaphragm muscles from 1-year-old wild-type, *KLF10*^{-/-}, mdx, and *KLF10*^{-/-}/mdx mice were immunostained with an anti-fibronectin antibody and fluorochrome-conjugated secondary antibody. The images are representative of analyses of six mice of each genotype. Scale bar = 50 μ m.

percentage muscle fiber area in *KLF10*^{-/-} muscle was significantly reduced compared with wild-type muscle ($84.9\% \pm 0.52\%$ versus $89.1\% \pm 0.81\%$). The percentage of muscle fiber area in mdx diaphragm was also reduced compared with wild-type diaphragm muscle ($83.1\% \pm 1.3\%$ versus $89.1\% \pm 0.81\%$). The percentage muscle fiber area in *KLF10*^{-/-}/mdx diaphragm muscle was also significantly reduced ($53.6\% \pm 1.31\%$) compared with wild-type muscle. Moreover, the percentage area composed of muscle fibers in *KLF10*^{-/-}/mdx diaphragm was significantly reduced compared with mdx diaphragm. Therefore, deletion of the *KLF10* gene in the background of wild-type and mdx genotypes significantly reduced contractile muscle tissue area relative to whole muscle area.

Functional Assessment

To determine whether the loss of *KLF10* caused effects on muscle function, grip strength was measured in mice of each genotype (Figure 9). *KLF10*^{-/-} mice displayed the same

grip strength as wild-type mice. The mdx mice, both female and male, had reduced grip strength compared with wild-type mice. More important, *KLF10*^{-/-}/mdx displayed significantly reduced grip strength compared with mdx mice. Female and male strengths were reduced by 26.6% and 29.3%, respectively, compared with mdx mice and 48.2% and 48.7%, respectively, compared with wild-type mice. These results further establish a causative link of loss of *KLF10* with increased fibrosis and loss of muscle function, particularly in dystrophic muscle.

Discussion

Many muscular dystrophies are partly characterized by increased deposition of extracellular matrix components, such as collagen and fibronectin, in response to chronic muscle damage. This fibrosis has important negative effects on normal muscle structure, function, and regenerative processes.^{5,6,45} Similar to the response to damage in other tissues, chronic skeletal muscle tissue damage results in

Figure 7 Immunodetection of embryonic myosin heavy chain (EMyHC) and laminin. Cryosections of tibialis anterior (TA) and diaphragm muscles from 1-year-old wild-type (WT), *KLF10*^{-/-}, mdx, and *KLF10*^{-/-}/mdx mice were immunostained with anti-EMyHC and anti-laminin antibodies, followed by fluorochrome-conjugated secondary antibodies. **Arrows** indicate muscle fibers containing EMyHC. The images are representative of analyses of six mice of each genotype. Scale bar = 50 μ m.

Figure 8 Quantitation of muscle fiber cross-sectional area and fibrosis. **A** and **B**: The average muscle fiber cross-sectional areas for tibialis anterior and diaphragm muscles, respectively, from 1-year-old wild-type (WT), *KLF10*^{-/-}, mdx, and *KLF10*^{-/-}/mdx mice were determined. **C** and **D**: The average percentage of muscle tissue composed of muscle fibers was also determined for each muscle from each genotype. Means of each genotype were compared with wild-type, *KLF10*^{-/-}, and mdx mice. Data are expressed as means $\pm$ SEM. $n = 6$ (**A–D**). * $P < 0.05$ versus WT; † $P < 0.05$ versus *KLF10*^{-/-}; ‡ $P < 0.05$ versus mdx.

elevated TGF- β signaling activity, and it is this activity that appears to initiate and sustain the fibrotic response to such tissue damage.^{10,11}

The *KLF10* gene is transcriptionally activated as part of the early response to activation of the TGF- β signaling pathway.²¹ Because *KLF10* is a transcriptional effector of TGF- β signaling, a *KLF10* gene knockout mouse model was investigated for its role in regulating normal muscle tissue structure in wild-type mice and for its role in muscle tissue structure in the mdx mouse model of Duchenne

muscular dystrophy. The *KLF10* gene was expressed at basal levels in wild-type TA and diaphragm muscles, and its expression was increased in mdx muscles. The increase was modest in TA muscles and more pronounced in the diaphragm. The absence of *KLF10* gene expression significantly increased *Colla1* and fibronectin gene expression and protein deposition, particularly in the mdx diaphragm, exacerbating the fibrotic phenotype.

Expression of genes encoding the TGF- β signaling effectors SMAD2, 3, 4, and 7 was assessed in the context of

Figure 9 Assessment of muscle function by grip strength. Wild-type (WT), *KLF10*^{-/-} (K), mdx (M), and *KLF10*^{-/-}/mdx (KM) mice were measured for grip strength. Five female and five male mice of each genotype were assessed using five repetitions for each mouse. Means of grip strength of each genotype were compared with wild-type, *KLF10*^{-/-}, and mdx mice. Data are expressed as means $\pm$ SEM. $n = 5$. * $P < 0.05$ versus WT; † $P < 0.05$ versus *KLF10*^{-/-}; ‡ $P < 0.05$ versus mdx.

KLF10 gene expression in wild-type and mdx mice. SMAD2 protein was detected in TA muscle, regardless of the genotype tested. However, SMAD2 protein was absent in wild-type and mdx diaphragm and present in *KLF10*^{-/-} muscles, indicating that *KLF10* may repress *SMAD2* gene expression in the diaphragm. SMAD3 protein was detected in *KLF10*^{-/-}/mdx TA and diaphragm muscles, suggesting that *KLF10* may also repress *SMAD3* gene expression in these muscles. Furthermore, phosphorylated SMAD2/3 was detected in nuclei of *KLF10*^{-/-} and *KLF10*^{-/-}/mdx diaphragm muscle. These results are important in light of the established role of SMAD2 and SMAD3 in the development of tissue fibrosis.⁶ SMAD4 protein was more abundant in diaphragm versus TA muscle protein extracts, but no differences corresponding to genotypes were readily observed. SMAD7 protein was detected in *KLF10*^{-/-} and *KLF10*^{-/-}/mdx diaphragm muscle protein extracts and was not detected in the other extracts. This pattern of *SMAD7* gene expression suggests that *KLF10* represses *SMAD7* gene expression, and this result is in agreement with previous findings.³² No discernible change in TGF- β R1 protein content was observed in any of the protein extracts tested. Overall, these gene expression studies indicate that *KLF10* gene expression is increased in regenerating (EMyHC-positive) muscles that show signs of fibrosis (eg, mdx diaphragm). However, the loss of *KLF10* gene expression in these same muscles resulted in dysregulation of SMAD gene expression. SMAD2 and SMAD3, which are positive effectors of TGF- β signaling, were increased in mdx muscles in the absence of *KLF10*. Therefore, as an immediate early gene of TGF- β signaling, *KLF10* may, at least transiently, repress *SMAD2* and *SMAD3* gene expression and thereby modulate the fibrotic response to muscle damage.

The dysregulation of SMAD gene expression in the absence of the *KLF10* gene correlated with increased fibrosis. Marked collagen and fibronectin gene expression and protein deposition were detected in the mdx diaphragm and *KLF10*^{-/-}/mdx TA and diaphragm muscles. Furthermore, fibrosis was increased in *KLF10*^{-/-}/mdx muscles compared with mdx. The altered patterns of SMAD gene expression in the absence of *KLF10* are partially in agreement with previous findings in other tissues. Similar to previous results, *KLF10* appears to repress *SMAD7* gene expression.³² However, it has been reported that *KLF10* increased expression of PAI-1 and *SMAD2*, but not *SMAD3* or *SMAD4*, in osteoblasts.³² The results presented herein indicate that *KLF10* regulation of *SMAD2* and *SMAD3*, but not *SMAD4*, may reflect differential tissue-specific TGF- β -induced signaling pathways, mediated by *KLF10*. Nonetheless, the absence of *KLF10* has been correlated with fibrosis in other striated muscle tissue. For example, *KLF10*-deficient mice display cardiac hypertrophy with increased fibrosis.⁴⁰

A reduction in average muscle fiber cross-sectional area was observed in mdx versus wild-type and in *KLF10*^{-/-}/mdx versus *KLF10*^{-/-} TA and diaphragm muscles. The loss of *KLF10* alone did not cause a significant reduction in average

muscle fiber size. This latter result is not in direct agreement with a recent study that reported the effect of loss of *KLF10* on muscle fiber size in mouse extensor digitorum longus and soleus muscles.⁴⁶ In this study, loss of *KLF10* resulted in increased mean muscle fiber size in both muscles. These results indicated that loss of *KLF10* led to muscle fiber hypertrophy, the mechanism of which is unclear. The study did not examine hypertrophy of specific muscle fiber types. It is important to recognize basic differences in experimental design between this later study and the one reported herein. Perhaps most important, the former study used 3-month-old mice, and the experiments reported herein used 1-year-old mice. This age was considered a time point at which skeletal muscle had sufficient time to develop fibrosis under normal levels of activity. It is not known at what age similar fibrotic changes can first be detected. The differences in results may reflect the role of *KLF10* in muscle structure and homeostasis over time. It is possible that hypertrophy is an early response to loss of *KLF10*. This response may be transient, evident at 3 months of age, and decline between 3 months and 1 year.

Such changes in muscle fiber morphometry may accompany other changes in muscle histology and function over time because of loss of *KLF10*. The loss of muscle fiber size over time from 3 months to 1 year may be accompanied, or because of, a commensurate increase in fibrotic tissue. This is evident in the results reported herein. One-year-old *KLF10*^{-/-} TA and diaphragm muscles displayed increased fibrosis relative to age-matched wild-type controls. One-year-old *KLF10*^{-/-}/mdx muscles also had increased fibrosis relative to mdx mouse muscles. These changes in muscle architecture correlate with reduced muscle function, as determined by measurements of grip strength. Also, the morphometric results were supported by assays of hydroxyproline content, reflecting collagen deposition, in these muscles. The mdx and *KLF10*^{-/-}/mdx diaphragm muscles had greater hydroxyproline levels than their respective controls (wild-type and mdx muscles, respectively). No significant difference in hydroxyproline content was detected between wild-type and *KLF10*^{-/-} muscles, yet fibrosis in *KLF10*^{-/-} diaphragm muscle was suggested by a significant decline in the percentage of muscle fiber area within sections of the muscle. This is likely because of other ECM components, such as fibronectin, contributing to fibrosis and relative loss of contractile tissue area. Nevertheless, the effect of the loss of *KLF10* was most pronounced in the mdx diaphragm, a muscle that exhibits fibrosis and significant muscle fiber degeneration and regeneration. Therefore, the results reported herein indicate that *KLF10* moderates fibrosis in the context of chronic muscle degeneration and regeneration.

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