Lack of Smad3 signaling leads to impaired skeletal muscle regeneration

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Ge X, Vajjala A, McFarlane C, Wahi W, Sharma M, Kambadur R. Lack of Smad3 signaling leads to impaired skeletal muscle regeneration. Am J Physiol Endocrinol Metab 303: E90–E102, 2012. First published April 24, 2012; doi:10.1152/ajpendo.00113.2012.—Smad3 is a key intracellular signaling mediator for both transforming growth factor-β and myostatin, two major regulators of skeletal muscle growth. Previous published work has revealed pronounced muscle atrophy together with impaired satellite cell functionality in Smad3-null muscles. In the present study, we have further validated a role for Smad3 signaling in skeletal muscle regeneration. Here, we show that Smad3-null mice had incomplete recovery of muscle weight and myofiber size after muscle injury. Histological/immunohistochemical analysis suggested impaired inflammatory response and reduced number of activated myoblasts during the early stages of muscle regeneration in the tibialis anterior muscle of Smad3-null mice. Nascent myofibers formed after muscle injury were also reduced in number. Moreover, Smad3-null regenerated muscle had decreased oxidative enzyme activity and impaired mitochondrial biogenesis, evident by the downregulation of the gene encoding mitochondrial transcription factor A, a master regulator of mitochondrial biogenesis. Consistent with known Smad3 function, reduced fibrotic tissue formation was also seen in regenerated Smad3-null muscle. In conclusion, Smad3 deficiency leads to impaired muscle regeneration, which underscores an essential role of Smad3 in postnatal myogenesis. Given the negative role of myostatin during muscle regeneration, the increased expression of myostatin observed in Smad3-null muscle may contribute to the regeneration defects.

myostatin; satellite cell; mitochondria

SMAD3 belongs to a family of nine proteins named Smad proteins, which function as key intracellular signaling mediators for the transforming growth factor-β (TGF-β) superfamily (6, 52), a large group of extracellular growth factors modulating diverse biological processes and tissue homeostasis (24, 62). Mutagenesis of the Smad3 gene in mice provides an in vivo model for the analysis of Smad3 function. To date, three different Smad3-null mice have been developed, by targeted disruption of exon1, exon 2, and exon 8 (10, 72, 74). Smad3-null (Smad3(exon 8)−/−) mice, which were used in this study, express a truncated SMAD3 protein lacking the SSVS consensus phosphorylation site and an L3 loop (72), which are essential domains required for the activation of Smad3 by cell surface receptors (32). Although Smad3-null mice are viable and can survive to adulthood, they usually die between 1 and 8 mo due to inflammation in a variety of organs, including the nasal mucosa, stomach, pancreas, colon, and small intestine (72). Smad3 deficiency leads to impaired TGF-β regulation of T cell activation. This major immune deficiency increases the susceptibility of Smad3-null mice to bacterial infections and results in the chronic inflammation (72). Due to chronic inflammation, Smad3-null mice are also more susceptible to inflammation-associated cancer (36–37).

Although extensive studies have established the importance of Smad3 as a downstream mediator of TGF-β superfamily members, relatively very little is known about the specific functions of Smad3 in skeletal muscle growth, and in fact, results to date are controversial (16, 55). A report by Sartori et al. (55) has used RNAi techniques to knock down Smad3 expression in adult muscles. In this study, muscle hypertrophy was observed in Smad3 knockout mice (55), indicating a negative role for Smad3 in the regulation of postnatal muscle growth. However, in this study, a trace amount of Smad3 protein was still found in muscle fibers (55). In contrast to the results of Sartori et al., recently published work suggests that Smad3 is essential for normal skeletal muscle growth and that Smad3-null mice have profound muscle atrophy (16). Molecularly, it was determined that the muscle atrophy in Smad3-null mice was due to upregulated myostatin expression (16). Myostatin, a TGF-β superfamily member, is a potent negative regulator of skeletal muscle growth (22, 45) that controls myogenesis through inhibiting myoblast proliferation (42, 66, 71), differentiation (21, 26, 42, 53), as well as stellate cell (SC) activation and self-renewal (40, 43). In addition to regulating SC function, myostatin also inhibits protein synthesis and promotes sarcomeric protein degradation in skeletal muscle through the ubiquitin proteasome system (31, 33–34, 44, 69).

To further characterize the role of Smad3 in the regulation of postnatal myogenesis, we have induced muscle injury in Smad3-null mice through intramusculature notexin injection and assessed subsequent muscle regeneration histologically, molecularly, and metabolically. Given the increased myostatin expression and impaired SC functionality in Smad3-null mice (16), we hypothesized that Smad3-null muscle would regenerate poorly. Consistent with our hypothesis, Smad3-null mice showed impaired muscle regeneration in response to notexin administration, evident by dramatic loss of muscle weight, profound decline in the size of regenerated myofibers, and reduced centrally located myonuclei. Impaired inflammation, less myoblast activation, and reduced newly formed myofibers were observed in Smad3-null regenerating the tibialis anterior (TA) muscle. Moreover, the regenerated TA muscle from Smad3-null mice showed significantly decreased oxidative enzyme activity, due to defects in mitochondrial biogenesis. In
addition, loss of Smad3 also led to reduced formation of scar tissue during muscle regeneration.

MATERIALS AND METHODS

Animals. Heterozygote Smad3-null mice were kindly gifted by Walter Wahli (University of Lausanne, Lausanne, Switzerland) and were bred and genotyped according to the previously published protocol (72). Mice were maintained on standard chow diet at a constant temperature (20°C) under a 12:12-h artificial light-dark cycle with unlimited access to water. All experiments were performed according to the approved protocols of the Institutional Animal Care and Use Committee, Singapore.

Muscle injury. Six-week-old male mice were anesthetized by intraperitoneal injection of a mixture of 10 mg/ml ketamine and 1 mg/ml xylazine at 0.1 ml/10 g body wt. Fifteen microliters of Notexin (10 μg/ml in 0.9% NaCl; Latioxan, Rosans, France) were injected in the TA muscle of the left leg, using a 28-gauge syringe (Hamilton, Whittier, CA). The right TA was used as an uninjected (uninjured) control. TA muscles were harvested and weighed on days 1, 2, 3, 7, and 30 after injury (n = 3/day).

Histological assessment of muscle regeneration. Serial cross sections (8 μm) were cut from the midbelly of the muscle for histological, immunohistochemical, and metabolic staining. Hematoxylin and eosin (H&E) staining and Van Giesen’s staining were performed according to the manufacturer’s instructions (Merck, Rahway, NJ). Images were captured using the Leica CTR 6500 microscope, equipped with the Leica DFC 310 FX camera (Leica, Wetzlar, Germany) and Image Pro Plus software (Media Cybernetics, Bethesda, MD). H&E staining was used for muscle fiber cross-sectional area (CSA) measurements according to Zeng et al. (73). Five images (×10) around the lesion area from each sample (n = 3) were captured, and the CSA of the centrally nucleated myofibers within the field were measured. Van Giesen’s stain was used for fibrotic tissue quantification according to Luz et al. (35) and calculated as the ratio of the fibrotic area to the total muscle CSA.

Immunohistochemistry. Immunohistochemical staining on muscle sections was performed as described previously (41). In brief, frozen muscle sections (8 μm) were blocked in 0.2% Triton X-100, 0.2% BSA, and 10% normal sheep serum (NSS) in PBS for 1 h at room temperature, followed by incubation with primary antibodies diluted in blocker overnight at 4°C. After fixation with 10% buffered formalin for 5 min, the sections were incubated with secondary antibodies and/or tertiary antibodies in 0.2% BSA and 5% NSS in PBS-T for 1 h. After being washed with PBS, the sections were counterstained with DAPI (1:1,000; Invitrogen-Molecular Probes, Eugene, OR) and mounted with ProLong Gold antifade reagent (Invitrogen-Molecular Probes). Mouse IgG1 isotype (DAKO, Carpenteria, CA) was used as a negative control to detect nonspecific background staining.

Succinate dehydrogenase and α-glycerophosphate dehydrogenase staining. Succinate dehydrogenase (SDH) and α-glycerophosphate dehydrogenase (α-GPD) activities were measured using a colorimetric assay, as described by Masuda et al. (38) with slight modification. In brief, for SDH staining, sections were air-dried for 30 min and then incubated in prewarmed PBS buffer containing 50 mM sodium succinate and 0.6 mM nitroblue tetrazolium (NBT) (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C. For α-GPD staining, the sections were incubated in prewarmed 0.2 M Tris-HCl buffer (pH 7.4) containing 9.3 mM rac-glycerol 1-phosphate disodium salt hexahydrate (Sigma-Aldrich) and 0.24 mM NBT for 45 min at 37°C after air-drying. The reaction was terminated by rinsing the sections with distilled water thoroughly. After dehydration and clearance by xylene, the sections were mounted using DPX (Sigma-Aldrich). Enzyme activities were expressed as the mean optical density (OD) value of all pixels within each fiber (n = 1,000) using Image J software (National Institute of Health, Bethesda, MD), as previously described (20). For uninjured muscle, the mean OD value for the whole TA cross sections was measured.

RNA preparation and quantitative real-time PCR analysis. Isolation of total RNA from TA muscle was performed using TRIzol reagent (Invitrogen-Molecular Probes), according to manufacturer’s instruction. Subsequent cDNA synthesis was conducted using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). SsoFast EvaGreen supermix (Bio-Rad) and the CFX96 Real-Time System (Bio-Rad) were used for quantitative real-time PCR (qPCR) reactions. Gene expression fold change was calculated using the ΔΔCt method, normalized against glyceraldehyde-3-phosphate dehydrogenase (Gapdh) gene expression. The sequences of oligonucleotides used in this study were as follows: CollagenIVal (forward) (F 5'-AGA CAT GCT CAG CTT GGA TAC-3') and (reverse) (R 5'-5'-AGC TGG CCG AGA ATT TCA CC-3'); CollagenIIVal (F 5'-AGG GAG TCC CAG AGA TTG TGT CTC CTC TAC AGA CC-3') and (reverse) (R 5'-AGA CTT GGC TTG TGG CAG CCC AGG-3'); myogenin (F 5'-GAG CGC CTC CCG TAC TAC AGA CC-3') and (reverse) (R 5'-TCC CCC AGC CTT GCC TCC CC-3'); mitochondrial transcription factor A (TFAM) (F 5'-GGG GCT GTC GCC CCG AGG AAC TTG TTG ATG-3') and (reverse) (R 5'-GAT TG TCC CCA GGT GTG CCG CCC AGG-3'); CollagenI (F 5'-CTG GCC GAT CTC TGC TAC AGA CC-3') and (reverse) (R 5'-GAG TGG CAG TGG CAC AGG CCA-3'); CollagenIII (F 5'-CTG GCC GAT CTC TGC TAC AGA CC-3') and (reverse) (R 5'-GAG TGG CAG TGG CAC AGG CCA-3').

Western blot. Protein extracts from TA muscles were made in protein lysis buffer [50 mM Tris, pH 7.6, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN)]. Twenty micrograms of total proteins were resolved by SDS-PAGE (4–12% gradient; Invitrogen-Molecular Probes) and transferred to a nitrocellulose membrane. Following blocking with 5% milk in TBS-T, the membrane was incubated with anti-TFAM primary antibody (1:400; Santa Cruz Biotechnologies, Santa Cruz, CA) and secondary antibody goat anti-rabbit IgG conjugated with horseradish peroxidase (1:5,000; Bio-Rad) in 5% milk in TBS-T. Immuneactivity was detected using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer, Waltham, MA). Band density was analyzed using the Quantity One imaging software (Bio-Rad).

Collagen concentration analysis. TA muscle samples were acid-hydrolyzed in 5 M HCl (1 ml 5 M HCl/10 mg of wet muscle wt) at 130°C for 12 h. Two aliquots for each sample (50 μl/aliquot) were taken for the hydroxyproline assay, using the method adapted from Switzer and Summer (63). Collagen concentration (μg/mg) of the TA muscle was calculated based on hydroxyproline content, assuming that collagen contains 13.4% hydroxyproline by weight (7).

Statistical analysis. Statistical significance between groups was assessed using unpaired two-tailed Student’s t-test, and results were considered significant at P < 0.05 (*), P < 0.01 (**), or P < 0.001 (***) . Results were expressed as means ± SE.

RESULTS

Smad3-null injured TA muscle showed reduced muscle weight after regeneration. The TA muscle of the left hindlimb of Smad3-null and wild-type mice was injected with notexin and allowed to regenerate for 30 days. TA muscle from the right hindlimb was used as a contralateral uninjured control. TA muscle weights were measured 1, 2, 3, 7, and 30 days after notexin injection, along with body weights. Relative muscle
weight change was calculated as the muscle weight change (between uninjured and injured muscle) normalized to 100 g of body wt. Figure 1A demonstrates the relative muscle weight change from day 1 to day 30 after injury in both genotypes. During the early phase of regeneration, both genotypes showed a similar pattern of muscle weight change (Fig. 1A). An increase in the relative weight of the injured TA muscle from day 1 to day 2 was found in both genotypes, which is attributed to the acute inflammatory response (9). On day 7, injured TA muscle weights were reduced compared with uninjured controls in both genotypes (Fig. 1A).

However, significant differences between the two genotypes were found on day 3 and day 30. On day 3, enhanced muscle weight was maintained in the wild-type injured muscle (0.08 ± 0.03%), whereas the Smad3-null muscle weight decreased (−0.01 ± 0.002%), compared with contralateral controls. This suggests reduced edema and inflammation response in Smad3-null mice on day 3. On day 30, wild-type TA muscle showed an increase in net weight (15.07 ± 1.48 mg) compared with respective contralateral controls (Fig. 1B). In contrast, the regenerated TA muscles from Smad3-null mice were lighter in weight (−5.80 ± 2.40 mg) compared with their respective uninjured controls (Fig. 1B). When muscle weights were normalized to body weight, a similar pattern was observed (Fig. 1C). Taken together, these data are indicative of impaired skeletal muscle regeneration in Smad3-null mice.

**Histological analysis during the early phase of Smad3-null mice muscle regeneration.** As demonstrated in Fig. 2A, notexin treatment induced complete and extensive muscle degeneration in both genotypes 1 day after notexin injection. Infiltration of mononuclear cells and edema were observed at day 1. However, lower numbers of mononuclear cells were seen in Smad3-null muscle compared with wild-type controls 1 day after notexin injection (Fig. 2A). On day 2, wild-type injured muscles were characterized by increased edema compared with muscles on day 1. However, Smad3-null injured muscle on day 2 had less edema compared with wild-type muscle on day 2. During day 2 and day 3, the inflammatory response extended and necrotic myofibers, which were hyperstained, were clearly seen. Necrotic myofibers phagocytosed by macrophages were also observed from day 1 to day 3 (Fig. 2A). Newly formed myofibers, characterized by centrally located nuclei and small fiber size, were visible on day 7 in TA muscles from both genotypes.

**Reduced inflammatory response in Smad3-null mice.** During the initial phase of muscle regeneration, large numbers of inflammatory cells, mainly neutrophils and macrophages, are recruited from circulation to the damaged muscle (58). Importantly, histological analysis during early muscle regeneration found reduced mononuclear cell infiltration in injured Smad3-null TA muscle compared with wild-type controls (Fig. 2A). Subsequent immunohistochemical analysis (Fig. 2, B and C) using an anti-Mac-1 antibody to identify macrophages and neutrophils revealed that Smad3-null muscle had significantly reduced inflammatory cells at day 2 and day 3 following injury (Fig. 2, B and C), suggesting that loss of Smad3 leads to decreased recruitment of inflammatory cells during muscle regeneration.

**Profound reduction in regenerated myofiber size in Smad3-null TA muscle after injury.** Morphological analysis, using H&E staining, was conducted to quantitatively evaluate muscle regeneration 30 days after injury (Fig. 3A). One of the prominent morphological differences between the two genotypes, after 30 days of injury, was that the regenerated fibers in Smad3-null muscle were significantly smaller than that of wild-type (Fig. 3A). The fiber size of the regenerated myofibers was quantified and represented by the mean CSA. The mean CSA of regenerated myofibers was ~98 ± 4.5% of its contralateral counterpart in wild-type mice at day 30 postinjury (Fig. 3B). This demonstrated that wild-type regenerated myofibers were comparable in size to that of uninjured controls. However, in Smad3-null mice, the mean CSA of regenerated fibers was only ~44 ± 8.0% of its uninjured control (Fig. 3B). Regenerated myofibers are characterized by the presence of centrally located myonuclei (4). Interestingly, we found that, in wild-type regenerated muscle, multiple (2–4) centrally located myonuclei were often observed in

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**Fig. 1.** Decreased regenerated tibialis anterior (TA) muscle weight in Smad3-null mice 30 days after injury. A: relative muscle weight change, calculated as the muscle weight change (the difference in weight between injured and control uninjured TA muscles) normalized to 100 g of body wt, at 1, 2, 3, 7, and 30 days after notexin injection in wild-type and Smad3-null mice. Results shown in the black dashed box on the graph are displayed in detail in the third panel (C). B: TA muscle weight change [the difference in the weight (mg) between injured and uninjured TA muscles] 30 days after notexin injection. C: relative TA muscle weight change 30 days after notexin injection. Values represent means ± SE. Statistical differences are indicated, *P < 0.05 (*) and **P < 0.01 (**); n = 3 in each group.
Fig. 2. Smad3 deficiency leads to a dramatically reduced inflammatory response in injured TA muscle. A: hematoxylin and eosin (H&E)-stained TA muscle cross sections from uninjured and injured muscles on days 1, 2, 3, and 7 after notexin injection in wild-type and Smad3-null mice. Infiltration of inflammatory cells (I), edema (E), necrotic fibers (N), and newly formed myofibers (*) is marked on the images. Scale bars represent 50 μm. B: anti-Mac-1 antibody was used to identify infiltrating macrophages and neutrophils in injured muscle cross sections. Scale bars represent 50 μm. White asterisks represent the Mac-1-positive cells. C: quantification of Mac-1-positive cells in wild-type and Smad3-null muscle cross sections at days 2 and 3 after notexin injection. Mac-1-positive cells were expressed as a percentage of the total nuclei (DAPI positive). Values represent means ± SE. Statistical differences are indicated, *P < 0.001 (**); n = 3 in each group.
each myofiber. However, in contrast, the majority of regenerated myofibers in Smad3-null muscle had only one central myonucleus (Fig. 3A). Subsequent quantification of central myonuclei revealed that 39 ± 2.6% of regenerated fibers in wild-type muscle had multiple central myonuclei (2–4), whereas only 6 ± 1.9% of the regenerated fibers in Smad3-null muscle had more than two central myonuclei (Fig. 3C).

Reduced number of myoblasts were found in Smad3-null regenerating muscle. Skeletal muscle regeneration is dependent on SC function (23, 39). In response to muscle injury, quiescent SCs become activated and proliferate before terminally differentiating to form multinucleated myotubes (1). Differentiated myotubes subsequently form nascent myofibers, which further undergo maturation and increase in size (50). Because
MyoD and myogenin are well-known markers of activated myoblasts and myogenic differentiation, respectively, we measured the mRNA transcript levels of both MyoD and Myogenin during the course of muscle regeneration (1, 2, 3, 7, and 30 days after notexin injection) by qPCR. Gene expression analysis suggested that MyoD expression increased to a peak level at day 3 after injury, progressively declined until day 7, and returned to levels comparable to that of uninjured control by day 30 postinjury in both genotypes (Fig. 4A). However, significantly lower MyoD expression was found in Smad3-null muscle at days 1, 3, 7, and 30 after injury compared with wild-type muscle (Fig. 4A). Expression of myogenin showed a similar pattern to that of MyoD during muscle regeneration in both genotypes (Fig. 4B). Peak myogenin expression was observed at day 3, with significantly reduced Myogenin mRNA expression detected in Smad3-null muscle at days 2, 3, and 7 after injury compared with wild-type muscle (Fig. 4B). To assess the number of activated SCs, we also performed MyoD immunohistochemical analysis (Fig. 4C) on injured TA muscle sections at day 3 (coinciding with peak MyoD mRNA expression). Consistent with the gene expression analysis, significantly reduced MyoD-positive cells were found in Smad3-null muscle at days 1, 2, 3, and 7 after injury compared to wild-type muscle (Fig. 4C).
muscle (Fig. 4C), indicating that less myoblasts were present in Smad3-null TA muscle after injury.

Reduced embryonic myosin heavy chain-positive nascent myofibers were found in Smad3-null regenerating muscle. Expression of eMyHC is a hallmark of muscle regeneration. Nascent myofibers express immature MyHC isoforms, such as eMyHC and neonatal MyHC, rather than adult MyHC (type I/IIA/IIB/ IIX) (70). To examine the formation of newly formed myofibers, eMyHC staining was performed on muscle sections at day 7 postinjury. At day 7, we observed a large number of eMyHC-expressing, centrally nucleated myofibers in wild-type TA muscle (Fig. 4D). However, only a few eMyHC-positive myofibers were found in Smad3-null regenerating muscle (Fig. 4D). In addition to immunohistochemical staining, we also analyzed eMyHC mRNA expression in regenerating muscles from both genotypes. Subsequent qPCR revealed a sharp increase in eMyHC expression in both genotypes on day 3, which gradually declined through day 30, where eMyHC expression was comparable to uninjured control (Fig. 4E). However, the peak eMyHC expression (day 3) and the expression of eMyHC at day 7 in Smad3-null injured TA muscle were reduced significantly compared with that of wild-type mice (Fig. 4E).

Metabolic properties of Smad3-null regenerating muscle. The metabolic properties of skeletal muscle determine muscle function, as well as metabolic substrate utilization. Here we studied the metabolic phenotypes of TA muscle at day 30 postinjury in both genotypes. Two important metabolic enzymes were analyzed in this study, SDH, a marker of aerobic (oxidative) capacity, and α-GPD for glycolytic potential (38). The activities of both enzymes in the regenerated myofibers (30 days postinjury) were demonstrated by histochemical reactions and quantified by image analysis. We observed uneven staining for SDH activity in regenerating TA muscles of both genotypes (Fig. 5A). However, a significant difference was found between the regenerated muscles from the two genotypes (Fig. 5A). Specifically, wild-type regenerated fibers showed strong SDH staining in the cytoplasm, whereas significantly less intense staining was observed in the regenerated myofibers in Smad3-null muscle (Fig. 5A), which suggested a lower level of oxidative enzyme activity. Quantitative analysis demonstrated that SDH activity in Smad3-null regenerating myofibers was ∼30% lower than that in wild-type myofibers (Fig. 5C). Even though slightly weaker staining for α-GPD activity was observed in Smad3-null mice, the difference between the two genotypes was not significant (P > 0.05) (Fig. 5, B and D). The GPD-to-SDH ratio is an index of metabolic substrate utilization (17). Figure 5E demonstrated that the GPD-to-SDH (glycolytic-to-oxidative) ratio was lower in Smad3-null muscle at day 30 postinjury compared with wild-type muscle. This shift in the GPD-to-SDH ratio in Smad3-null regenerating myofibers suggested enhanced reliance on glycolytic metabolism.

Oxidative metabolic pathways occur in the mitochondria of myofibers, and mitochondrial biogenesis is an important event for muscle regeneration (68). We hypothesized that lower oxidative enzyme activity in Smad3-null regenerating TA muscle might be due to impaired mitochondrial biogenesis. Therefore, we analyzed the mRNA expression of TFAM and NRF1, two master regulators of mitochondrial biogenesis (67), in regenerating TA muscles from both genotypes. As can be seen in Fig. 5F, mRNA expression of both TFAM and NRF1 was downregulated in Smad3-null TA muscle after 30 days of muscle regeneration. Western blot analysis confirmed the reduced expression of TFAM in Smad3-null regenerating muscle (Fig. 5G). However, we did not observe a significant difference in the protein level of NRF1 in the regenerating TA muscles from the two genotypes (data not shown).

Decreased scar tissue in Smad3-null regenerating muscle. Previous studies show that remodeling of extracellular matrix (ECM) during muscle regeneration begins at ~14 days postinjury (56). Excessive growth of ECM and deposition of collagen gives rise to scar tissue formation within the injured muscle tissue, which impairs the full recovery of muscle function (30). Van Giesen’s stain was used to examine fibrotic tissue within the regenerating muscle at day 30 after notexin injection (Fig. 6A). Quantification of fibrotic tissue, by morphometric analysis, suggested that Smad3-null mice had significantly reduced interstitial fibrotic tissue in regenerating muscle compared with wild-type mice (7 ± 0.06 compared with 20 ± 1.79%, respectively) (Fig. 6B). Because collagen is the most abundant structural component in skeletal muscle ECM (64), we also measured the total collagen content in regenerating TA muscle via hydroxyproline assay. Thirty days after notexin injection, collagen accumulation in Smad3-null TA muscle after regeneration was significantly lower compared with wild-type regenerated muscle (Fig. 6C). Fibrillar collagen type I and III and nonfibrillar collagen type IV are the predominant collagen isoforms in skeletal muscle (3). Therefore, the mRNA levels for collagen Iα1, collagen IIIα1, and collagen IVα1 were also measured by qPCR. The results indicated that Smad3-null regenerating TA muscle had significantly decreased expression of collagen Iα1, collagen IIIα1, and collagen IVα1 (Fig. 6D).

Consistent upregulation of myostatin expression in Smad3-null TA muscle during the course of muscle regeneration. Myostatin plays a negative regulatory role in muscle regeneration, thus loss of myostatin results in improved muscle regeneration (41). Therefore, the endogenous expression of myostatin during the course of muscle regeneration was determined by qPCR (Fig. 7). As can be seen in Fig. 7, during the regeneration process, the expression of myostatin in Smad3-null TA muscle was consistently higher compared with that in wild-type muscle (Fig. 7). In wild-type injured TA muscle, myostatin mRNA expression steadily increased after injury, peaked at day 2, and then declined until day 7 before returning to the uninjured control level (Fig. 7). However, in Smad3-null injured TA muscle, myostatin expression dropped sharply at day 1 from the uninjured control level and subsequently increased until day 3. Much akin to the pattern observed in wild-type muscle, myostatin expression then decreased until day 7 and finally returned to the uninjured control level at day 30 in Smad3-null mice (Fig. 7).

DISCUSSION

In this study, we used notexin-induced muscle injury to analyze the regeneration capacity of Smad3-null muscle, with the overall aim of further understand the role of Smad3 signaling in the regulation of postnatal myogenesis. Our results suggest that Smad3 is essential for normal muscle regeneration, since Smad3 deficiency resulted in defective muscle repair.

The role of Smad3 in adult muscle regeneration. During the early stage of muscle regeneration, significantly reduced mono-
cyte infiltration was observed in Smad3-null injured muscle (Fig. 2). This decreased inflammatory response in Smad3-null injured muscle could result from the impaired chemotaxis of Smad3-null monocytes. It is well-known that TGF-β is a potent chemoattractant for neutrophils (51); moreover, Smad3-null monocytes have an intrinsic impaired response toward TGF-β-induced chemotaxis (72). In agreement with the data presented here, reduced inflammatory response has been reported in skin excisional wounds (2) and cutaneous wounds of Smad3-null mice (15). Furthermore, reduced neutrophil infiltration was also found in the infarcted myocardium of Smad3-null mice during the early stage of the inflammatory response (8). Proinflammatory cytokines released by neutrophils recruit macrophages. A
A subpopulation of proinflammatory macrophages (CD68⁺) releases proinflammatory cytokines to promote inflammatory response and are also responsible for the phagocytosis of muscle debris. Once the necrotic tissue is cleared, proinflammatory macrophages undergo a phenotype switch to become anti-inflammatory macrophages (CD163⁺), which secrete anti-inflammatory cytokines such as interleukin-10 to terminate the inflammatory response. This anti-inflammatory population of macrophages is believed to promote muscle regeneration (46). Therefore, timely activation and repression of the inflammatory response is critically important for muscle regeneration. In fact, a previous study has shown that suppression of macrophages at the time of injury leads to incomplete regeneration (59). Moreover, blockade of anti-inflammatory macrophages leads to small regenerated myofiber size (65). Therefore, the defective inflammatory response to injury results in the accumulation of scar tissue.

Fig. 6. Decreased scar tissue formation in Smad3-null muscle after injury. A: representative images of Van Giesen’s-stained TA muscle cross sections, 30 days postinjury, from wild-type and Smad3-null mice. Scale bars represent 50 μm. B: quantification of interstitial fibrotic tissue by morphometric analysis. The percentage of fibrosis in each sample was calculated as the ratio of the fibrotic area to the total muscle CSA (%). Values represent means ± SE. C: total collagen content of regenerated TA muscles 30 days after notexin injection, as quantified by hydroxyproline assay. Values represent means ± SE. D: qPCR analysis of Collagen Iα1, Collagen IIIα1, and Collagen IVα1 expression in regenerated TA muscle from wild-type and Smad3-null mice (30 days after notexin injection). Graphs display fold change ± SE, normalized to Gapdh. Statistical differences are indicated, P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***); n = 3 in each group.
response found in Smad3-null skeletal muscle may contribute to the impaired muscle regeneration.

The weight and the size of regenerated myofibers in Smad3-null TA muscle were remarkably reduced compared with uninjured controls (Figs. 1 and 3). These defects clearly indicate reduced myogenesis, which we propose could be due to impaired SC activity. SCs play a pivotal role during muscle regeneration (61). In response to muscle injury, SCs are activated and migrate to the site of injury, in response to chemotactic signals released by macrophages, to furnish muscle precursor cells, which then proliferate and differentiate to give rise to nascent muscle fibers (5, 19, 61). Using MyoD as a marker for activated myoblasts, we observed significantly reduced myoblast populations in the damaged area of injured Smad3-null TA muscle (Fig. 4C). Previously published work from our laboratory has revealed that reduced SC number in Smad3-null muscle fibers is due to reduced SC self-renewal capacity. Moreover, in vitro experiments also revealed decreased proliferation capability and defective myoblast fusion in Smad3-null primary myoblasts (16). Normally, the pool of activated SCs present in muscle fibers provides sufficient myoblasts within the injured area for myogenesis. However, loss of Smad3 led to a reduction in the activated SC population recruited to the injured area in Smad3-null muscle, contributing to the impaired muscle regeneration. Furthermore, the majority of regenerated myofibers in Smad3-null mice only have one central nucleolus, indicating impaired myoblast fusion (Fig. 3). In support, previously published primary culture experiments revealed that Smad3-null myotubes are smaller, contain less number of myonuclei per myotube, and have reduced fusion index. In addition, several fusion-related genes, *BID Integrin*, *SHP-2*, and *Caveolin-3*, were also downregulated (16). Therefore, the impaired myoblast fusion intrinsic to Smad3-null myoblasts might explain the smaller size and reduced central myonuclei number of Smad3-null regenerated myofibers, thus contributing to incomplete muscle recovery.

In addition, we further analyzed the metabolic properties of regenerated muscle from both wild-type and Smad3-null genotypes mice through histochemical staining of SDH and α-GPD activity. SDH activity is an indicator of mitochondrial oxidative capacity (18) and tricarboxylic acid cycle activity in myofibers, whereas the levels of α-GPD in both cytosol and mitochondria reflect the activity of the glycerol-phosphate shuttle, and as such is considered to be a key enzyme in muscle fiber glycolysis (49). Our results demonstrated that Smad3-null TA muscle had significantly decreased levels of SDH activity compared with wild-type controls after 30 days of muscle regeneration, suggesting that regenerated Smad3-null muscle has reduced oxidative capacity (Fig. 5). Because differences in the muscle fiber type of regenerated muscle might be responsible for the variation in muscle metabolic profile, we compared the muscle fiber type composition between wild-type and Smad3-null regenerated muscles. However, subsequent immunohistochemical staining with antibodies against adult MyHC isotypes revealed no significant difference between the two genotypes (data not shown). Therefore, the significant decline in the oxidative enzyme activity of Smad3-null regenerated muscle was not due to loss of slow-twitch fibers or increased fast-twitch fiber number. Thus we suggest that impaired mitochondrial biogenesis in Smad3-null muscle during regeneration, as evident by reduced expression of the master regulator of mitochondrial biogenesis, TFAM (Fig. 5), may be responsible for the metabolic differences. TFAM is a key transcription factor responsible for both the replication and transcription of mitochondrial DNA (57); thus, its downregulation in Smad3-null regenerate muscle indicates defective mitochondrial biogenesis. Previous studies suggest an essential role for mitochondria in muscle regeneration, where mitochondria are required for myoblast proliferation and differentiation in addition to its primary function in energy metabolism (54, 60). In vivo muscle regeneration studies have indicated that expression of mitochondrial biogenesis-related genes is synchronized with the expression of myogenic genes, such as *MyoD* and *Myogenin* (13, 68). As such, blocking of mitochondrial protein synthesis during muscle regeneration leads to small myofibers (68). In addition, unpublished data from our laboratory has revealed that genes pertinent to mitochondrial biogenesis were upregulated at both transcriptional and translational levels in myostatin-null skeletal muscle compared with the wild type (N. Rashid, R. Kambadur, and M. Sharma, unpublished observation). Moreover, lack of myostatin also enhanced mitochondrial function and activity (S. Lokireddy, I. Wijesoma, S. Teng, S. Bonala, C. McFarlane, M. Sharma, and R. Kambadur, unpublished observation). Thus, increased *myostatin* expression (Fig. 7) and subsequent myostatin-mediated impairment of mitochondrial function may be responsible for the defective mitochondrial biogenesis and reduced oxidative enzyme activity observed in Smad3-null regenerated muscle. Therefore, impaired mitochondrial biogenesis could be one of the contributing factors behind the muscle regeneration defects in Smad3-null mice.

**Role of myostatin in the impaired muscle healing in Smad3-null mice.** Previously published work from our laboratory revealed that *myostatin* expression was upregulated in Smad3-null muscle (16). Furthermore, genetic inactivation of *myostatin* in Smad3-null mice rescued both muscle atrophy and SC functionality in Smad3-null mice (16). Taken together, these data underscore the involvement of myostatin in the impaired myogenesis observed in Smad3-null mice. In agreement with our previous publication, we observed consistent upregulation of *myostatin* mRNA expression during regeneration in Smad3-null injured TA muscle (Fig. 7). Therefore, we speculate that increased *myostatin* expression in Smad3-null regenerating TA
Unpublished data from our laboratory has revealed that microRNA-27a/b (miRNA-27a/b) negatively regulates myostatin expression at the posttranscriptional level, through a Smad3-dependent mechanism (A. V. Bulusu, H. Arigela, S. Lokireddy, X Ge, C. McFarlane, M. Sharma, and R. Kambadur, unpublished observation). Moreover, unpublished data revealed that loss of Smad3 resulted in decreased expression of miRNA-27a/b in skeletal muscle. Taken together, we hypothesize that Smad3 deficiency would result in reduced miRNA-27a/b expression, leading to the increased levels of myostatin and impaired skeletal muscle regeneration observed in Smad3-null mice.

The role of Smad3 in scar tissue formation during muscle regeneration. Remodeling of connective tissue is an essential step in muscle regeneration, and fibroblasts are the major mediators of fibrosis (56). Following muscle degeneration, mesenchymal fibroblasts are activated to become myofibroblasts. They proliferate, differentiate, and express high levels of α-smooth muscle actin, which is required for wound closure (48). The balance between fibrosis and myofiber regeneration determines the functional recovery of skeletal muscle (14). In fact, excessive accumulation of fibrotic tissue during muscle regeneration leads to scar tissue formation, which results in attenuated muscle strength and functional recovery (30). In this study, we found increased deposition of interstitial fibrous tissue in wild-type TA muscle at day 30 of regeneration compared with Smad3-null regenerated muscle (Fig. 6). Furthermore, gene expression analysis also suggested that genes encoding the major collagen isoforms in skeletal muscle (Collagen I, Collagen III, and Collagen IV) were downregulated in Smad3-null regenerated muscle (Fig. 6). In agreement with this, Smad3 has been previously shown to directly associate with the promoters of ECM-related genes, such as Collagen Iα1 and connective tissue growth factor, to initiate gene expression (28–29), as well as regulate the expression of ECM-related genes through controlling the biogenesis of miRNAs (11). Furthermore, loss of Smad3 also protects against tissue fibrosis in a number of diseases associated with kidney, liver, intestine, and skin (15, 27). Therefore, our data, together with previous studies, provide strong evidence to support an essential role of Smad3 in tissue fibrosis. Interestingly, recently published work has revealed an interaction between fibroblasts and SCs during muscle regeneration. Specifically, fibroblasts positively regulate SC expansion and promote muscle maturation, whereas ablation of fibroblasts leads to impaired muscle regeneration and smaller nascent myofibers (47). Importantly, previous studies have demonstrated that Smad3-null fibroblasts have impaired function, with reduced migratory activity in response to serum stimulation and defective myofibroblast trans-differentiation (12). In addition, Smad3-null fibroblasts have decreased proliferation and reduced profibrotic response in vitro in response to TGF-β stimulation (25). Therefore, impaired function of Smad3-null fibroblasts may also result in defective muscle regeneration in Smad3-null mice.

In summary, Smad3 plays a critical role in muscle regeneration, from the early inflammatory response through myogenesis and finally formation of fibrotic tissue. Based on the results from this study, we propose a model that supports a role for Smad3 in the regulation of SC function, myoblasts fusion, inflammatory response, and fibrotic tissue deposition during muscle regeneration (Fig. 8).

Fig. 8. Proposed model for the role of Smad3 in muscle regeneration. Smad3-null mice show defective muscle regeneration in response to muscle injury (myotrauma). Smad3 deficiency leads to a reduced inflammatory response during muscle regeneration, due to impaired chemotaxis of Smad3-null monocytes. Loss of functional Smad3 also results in defective SC function and myoblast fusion, possibly due to constant upregulation of myostatin expression during the course of muscle regeneration. Because Smad3 plays an essential role in skeletal muscle fibrosis, Smad3-null regenerating tissue has reduced fibrotic tissue deposition.

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DISCLOSURES

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS


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