

GENERAL ARTICLE

Spp1 (osteopontin) promotes TGF β processing in fibroblasts of dystrophin-deficient muscles through matrix metalloproteinases

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Abstract

Duchenne muscular dystrophy (DMD) is caused by mutations in the gene encoding dystrophin. Prior work has shown that DMD progression can vary, depending on the genetic makeup of the patient. Several modifier alleles have been identified including *LTBP4* and *SPP1*. We previously showed that *Spp1* exacerbates the DMD phenotype in the *mdx* mouse model by promoting fibrosis and by skewing macrophage polarization. Here, we studied the mechanisms involved in *Spp1*'s promotion of fibrosis by using both isolated fibroblasts and genetically modified mice. We found that *Spp1* upregulates collagen expression in *mdx* fibroblasts by enhancing TGF β signaling. *Spp1*'s effects on TGF β signaling are through induction of MMP9 expression. MMP9 is a protease that can release active TGF β ligand from its latent complex. In support for activation of this pathway in our model, we showed that treatment of *mdx* fibroblasts with MMP9 inhibitor led to accumulation of the TGF β latent complex, decreased levels of active TGF β and reduced collagen expression. Correspondingly, we found reduced active TGF β in *Spp1*^{-/-}*mdxB10* and *Mmp9*^{-/-}*mdxB10* muscles *in vivo*. Taken together with previous observations of reduced fibrosis in both models, these data suggest that *Spp1* acts upstream of TGF β to promote fibrosis in *mdx* muscles. We found that in the context of constitutively upregulated TGF β signaling (such as in the *mdxD2* model), ablation of *Spp1* has very little effect on fibrosis. Finally, we performed proof-of-concept studies showing that postnatal pharmacological inhibition of *Spp1* reduces fibrosis and improves muscle function in *mdx* mice.

Introduction

Mutations in *DMD*, the gene encoding for dystrophin protein, cause Duchenne muscular dystrophy (DMD), a disease characterized by progressive muscle deterioration and eventual cardiac

and pulmonary dysfunction (1,2). In DMD, insufficient levels of dystrophin protein cause contraction-induced damage to the sarcolemma, leading to myofiber death and inflammatory cell

Received: May 13, 2019. Revised: July 15, 2019. Accepted: July 18, 2019

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invasion. While muscle stem cells partially compensate for muscle cell loss, the repeated cycles of cell death and repair lead to accumulation of TGF β , accumulation of extracellular matrix (ECM), and replacement of muscle by fat and connective tissue.

ECM deposition (i.e. fibrosis) contributes significantly to the severity and rate of DMD disease progression. Support for this hypothesis was bolstered by the identification of latent TGF β binding protein 4 (LTBP4) as a modifier of DMD severity (3). LTBP4 belongs to a family of TGF β binding proteins that bind to both precursor and active TGF β ligands, regulating their release through proteolytic cleavage (4). LTBP4 was first identified as a DMD modifier through murine linkage studies, in which a 12 amino acid deletion in *Ltbp4* segregated with two pathogenic features of murine muscular dystrophy (3). This “risk allele” in the DBA/2 J background correlated with higher levels of TGF β activity, enhanced membrane permeability and increased fibrosis compared to the “protective allele” that retained the 12 amino acid sequence. This polymorphism is located in the proline rich hinge region that needs cleavage to release the sequestered TGF β ligand. Subsequently, several independent genotype/phenotype studies identified a risk haplotype in LTBP4 that correlated with a shorter ambulatory period and more severe cardiomyopathy in DMD patients (5–7). The deleterious human haplotype involves four non-synonymous amino acids (VTTT) in LTBP4, whereas the IAAM haplotype is protective, correlating with slower disease progression. The protein produced from the protective haplotype binds more TGF β and is associated with reduced TGF β signaling (5,8). Thus, TGF β -mediated fibrosis has a strong link to DMD disease progression.

Spp1 (Osteopontin) is another modifier of dystrophinopathies that is highly upregulated with dystrophic onset and progression in mice (*mdx*), dogs golden retriever muscular dystrophy (GRMD) and humans (DMD) (7,9–12). Spp1 (24) is a highly acidic, secreted protein that binds to integrins (13,14) or CD44 (15) to modify signaling or mediate cell adhesion to the ECM (16). Correlations between Spp1 levels and dystrophic disease severity have been demonstrated in mice (9,10), dogs (11) and humans (7,12), which lends support for its role as a DMD modifier and potential biomarker. Ablation of *Spp1* in *mdx* mice attenuated disease severity and was associated with reduced TGF β and fibrosis, as well as with increased muscle regeneration and strength (10). In humans with DMD, a single nucleotide polymorphism (SNP) in the *Spp1* promoter (rs28357094T > G-referred to as the “G allele”) correlated with earlier loss of ambulation and decreased grip strength (7,12). These disease indicators also correlated with increased *Spp1* mRNA expression compared to patients with the protective “T allele” (12). Not all subsequent reports reproduced these original findings; however, the studies that substantiated rs28357094T > G in *Spp1* as a modifier used cohorts that were more ethnically homogeneous than those studies that did not support the original finding (17). The modifier effect was even more pronounced in patients on steroids, which may be due to a glucocorticoid-receptor binding element in the *Spp1* promoter near the location of the modifier SNP (18). Thus, both LTBP4 and SPP1 are modifiers of dystrophinopathies that impact TGF β activity and fibrosis and exacerbate disease progression.

Studies examining the relationship between Spp1 and LTBP4/TGF β have observed a feed forward effect in the context of sarcolemmal repair of myofibers (19). Spp1 ablation correlated with reduced expression of TGF β and, conversely, increased activation of TGF β signaling correlated with increased Spp1. For example, muscles in *mdx* mice on the DBA/2 J background (carrying the LTBP4 risk allele that is associated with higher TGF β activity) showed higher Spp1 expression

than dystrophic mice expressing the protective allele (19). However, the molecular mechanisms and effects of Spp1 and TGF β cascades are still unknown with regards to promotion of fibrosis and pathological ECM remodeling, which is the subject of this investigation.

Our previous studies demonstrated that global *Spp1* ablation correlated with reduced fibrosis and decreased TGF β in dystrophic muscle (10); however, the cell type responsible for mediating these changes was not identified. *Spp1* ablation altered macrophage polarization toward M2c, but macrophage-derived TGF β 1 was unchanged, nor was there altered expression of any other macrophage-derived pro-fibrotic factor ((9) and unpublished data). Moreover, TGF β expression was not changed with Spp1 ablation when the total leukocyte population in *Spp1*^{-/-} *mdx* was compared to *mdx* muscles (Supplementary Material, Fig. S1). This result suggests that the reductions in TGF β observed in *Spp1*^{-/-} dystrophic muscles must derive from a cell type other than leukocytes.

Spp1 has been previously shown to promote fibroblast differentiation and induce upregulation of collagen I expression in pathological fibrosis associated with liver, skin and lung tissues (20–22). Here we identify resident muscle fibroblasts as an important source and target of the Spp1-TGF β pathway and dissect the mechanism whereby Spp1 interacts with fibroblasts to promote fibrosis in the context of dystrophic muscles. We demonstrate that Spp1 promotes fibroblast expression of MMP9 and increased TGF β processing to induce collagen expression in skeletal muscle fibroblasts. Furthermore, we show that Spp1 ablation in the setting of very high TGF β does not improve collagen deposition and fibrosis in dystrophic muscle, thus demonstrating that Spp1 acts upstream of TGF β to regulate fibrosis in muscular dystrophy. Postnatal pharmacological inhibition of Spp1 lowers TGF β in skeletal muscles, decreases collagen accumulation and ameliorates disease severity. These data support Spp1 as a beneficial therapeutic target in DMD-associated fibrosis.

Results

Autocrine induction of collagen I in fibroblasts by Spp1

To interrogate Spp1's influence on fibroblasts in promotion of fibrosis in dystrophic muscles, we studied primary fibroblasts isolated from *mdx* *Spp1*^{-/-} *mdx* muscles, which allowed us to examine the influence of exogenously added Spp1 on a pure population of fibroblasts, independent of Spp1 derived from other cell types. *Spp1*^{-/-} *mdx* fibroblasts were incubated with conditioned media (CM) from *Spp1*^{-/-} *mdx* or *Spp1*^{+/+} *mdx* primary fibroblasts for 24 hours. Following incubation, collagen I gene expression was quantified as a marker of fibrosis, since collagen I is the main component of fibrotic ECM in dystrophic muscles. Using fibroblast-derived CM ensured that the proper cell-specific post-translational modifications would be present on Spp1 added to the cells. We observed a significant upregulation of collagen I expression in cells incubated with *Spp1*^{+/+} *mdx* CM compared to *Spp1*^{-/-} *mdx* CM, supporting the hypothesis that fibroblast-derived Spp1 acts in an autocrine manner to promote collagen I expression by fibroblasts (Fig. 1A). We also observed increased expression of collagen 3 (another collagen upregulated in fibrosis) but not fibronectin in cells incubated with *Spp1*^{+/+} *mdx* CM (Supplementary Material, Fig. S2A and B).

To determine whether the effect of *Spp1*^{+/+} CM on collagen expression was Spp1 specific, we purified Spp1 from *Spp1*^{+/+} *mdx* CM by immunoprecipitation (Fig. 1B). Subsequently, primary *Spp1*^{-/-} *mdx* fibroblasts were incubated with the

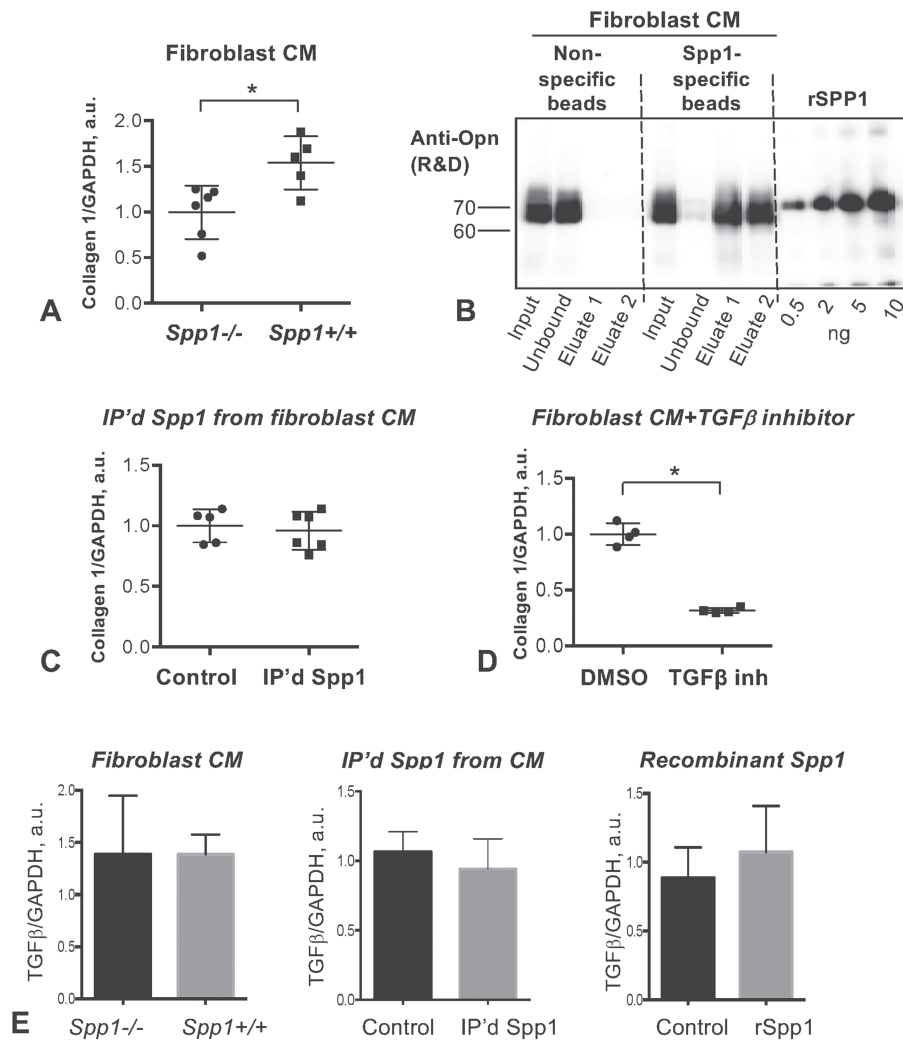


Figure 1. The autocrine effect of Spp1 on muscle fibroblast collagen expression is mediated by TGF β . (A) Primary *Spp1*^{-/-mdx} fibroblasts were incubated for 24 h with CM collected from either *Spp1*^{-/-mdx} or *Spp1*^{+/+mdx} fibroblasts, and expression of collagen I was assessed by RT-PCR. (B) Spp1 was purified from *Spp1*^{+/+mdx} fibroblasts CM by immunoprecipitation for use in subsequent experiments. Non-specific beads (left panel) were used as a negative control to insure specificity of immunoprecipitation by Spp1-specific antibodies (middle panel). The concentration of immunoprecipitated Spp1 was determined by comparison with a standard curve of rSpp1 (right panel) by densitometry. (C) Immunoprecipitated (IP'd) Spp1 was incubated with *Spp1*^{-/-mdx} fibroblasts, and collagen I expression was measured. (D) Expression of collagen I was significantly suppressed when *Spp1*^{-/-mdx} fibroblasts were incubated with CM of *Spp1*^{+/+mdx} fibroblasts, supplemented with the TGF β inhibitor (10 μ M of SB431542). (E) Twenty-four-hour incubation of *Spp1*^{-/-mdx} fibroblasts with *Spp1*^{+/+mdx} CM, IP'd Spp1 or rSpp1 did not upregulate expression of TGF β gene.

immunoprecipitated Spp1 (using the same concentration of Spp1 as was present in *Spp1*^{+/+mdx} CM, 50 ng/ml), and the effect on collagen I expression was evaluated. Surprisingly, we did not observe a similar increase in collagen expression in fibroblasts incubated with immunoprecipitated Spp1 as was observed in fibroblasts incubated with *Spp1*^{+/+mdx} CM, suggesting that Spp1's effect on fibroblast secretion of ECM is indirect (Fig. 1C).

We next sought to determine whether the effect of *Spp1*^{+/+mdx} CM on collagen I expression is mediated through TGF β , a known regulator of tissue fibrosis. To answer this question, primary *Spp1*^{-/-mdx} fibroblasts were incubated with *Spp1*^{+/+mdx} CM in the presence or absence of a TGF β inhibitor (10 μ M of SB431542) and were subsequently assessed for collagen I expression. As shown in Figure 1D, inhibition of TGF β in *Spp1*^{+/+mdx} CM significantly reduced fibroblast collagen induction, suggesting that TGF β mediates the effect of Spp1

on collagen expression in fibroblasts. To test whether Spp1 upregulates TGF β mRNA, primary fibroblasts were incubated with either: 1) *Spp1*^{+/+mdx} CM, 2) immunoprecipitated Spp1, or 3) commercially available recombinant Spp1 (rSpp1). After 24 h of incubation, TGF β mRNA was assayed by quantitative PCR and none of these treatments induced TGF β mRNA in the time frame of analysis (Fig. 1E). Thus, although the autocrine effect of Spp1 on collagen expression is mediated through a TGF β pathway, in this experimental context, Spp1 does not accomplish this task through direct induction of TGF β gene expression.

Spp1 is required for extracellular processing of TGF β

We next examined whether Spp1 influences posttranslational processing of TGF β . To address this question, CM from *Spp1*^{+/+mdx} and *Spp1*^{-/-mdx} fibroblasts was collected,

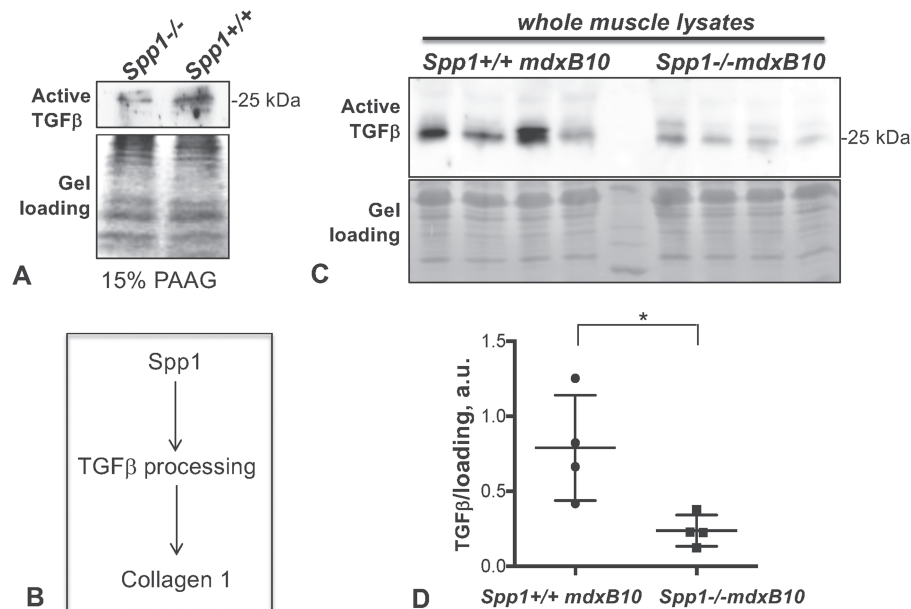


Figure 2. Extracellular processing of TGF β is decreased in the absence of Spp1. (A) Western blot analysis of concentrated CM from *Spp1*^{-/-mdx} and *Spp1*^{+/+mdx} fibroblasts revealed that levels of proteolytically processed (25 kDa) TGF β are decreased in CM lacking Spp1. Ponceau staining demonstrates equal loading of the concentrated CM. (B) Schematic representation of the hypothesis that Spp1 is required for normal extracellular processing of TGF β that in turn controls collagen I expression. (C) Western blot analysis of total muscle extracts from *Spp1*^{+/+mdxB10} and *Spp1*^{-/-mdxB10} mice demonstrates decreased levels of processed TGF β in the absence of Spp1. *n* = 4 of each genotype. Quantitative analysis of the western blot is shown in (D). Asterisk indicates statistical significance of *P* < 0.05. The nitrocellulose membrane was stained with ponceau after transfer and used to assess gel loading. Molecular weight markers were run in the middle lane and used to estimate TGF β size.

concentrated and analyzed by Western blotting for processed TGF β . This analysis revealed higher levels of the active form of TGF β (25 kDa) in *Spp1*^{+/+mdx} CM compared to *Spp1*^{-/-mdx} CM, suggesting that Spp1 indirectly influences TGF β cleavage and activation (i.e. processing) (Fig. 2A and B). *In vivo* support for this hypothesis was provided by analysis of whole muscle extracts whereby *Spp1*^{-/-mdxB10} muscles showed reduced levels of active TGF β compared to control *Spp1*^{+/+mdxB10} muscles (Fig. 2C and D). Taken together, our results indicate that Spp1 has an indirect effect on proteolytic processing of TGF β .

Spp1 regulates expression of MMP9 in fibroblasts

We next sought to identify whether Spp1 mediates its effects through upregulation of matrix metalloproteinases (MMPs), which have been previously associated with TGF β processing. MMP9 is highly elevated in dystrophic mdx muscles, and a relationship between Spp1 and induction of MMP9 expression was previously established (23). Based on these observations, we tested whether Spp1 ablation and subsequent reductions in MMP9 expression could be responsible for the decreased extracellular processing of TGF β in *Spp1*^{-/-mdx} fibroblasts. Consistent with prior *in vivo* observations, we found that primary fibroblasts treated with rSpp1 had increased levels of MMP9 mRNA (Fig. 3A) but did not show an increased expression of MMP2 (Supplementary Material, Fig. S2C). Moreover, we observed increased levels of both intracellular and secreted MMP9 protein in *Spp1*^{+/+mdx} compared to *Spp1*^{-/-mdx} fibroblasts (Fig. 3B). Thus, the effects of Spp1 on fibroblasts are mediated through MMP9 and its subsequent effects on TGF β processing to induce collagen.

It was previously demonstrated that Spp1 activates protein kinase B (AKT) signaling in hepatic stellate cells and promotes

fibrogenesis in the liver (24 upregulates collagen I via integrin α (V) β (3) engagement and PI3K/pAkt/NF κ B signaling). Consistent with this observation, we found that incubation with rSpp1 led to increased phosphorylation of AKT at Thr308 (Fig. 3C). Moreover, in the presence of the AKT inhibitor HY-15431 MedChemExpress (MCE), expression of MMP9 was significantly reduced, suggesting that Spp1 regulates expression of MMP9 via AKT activation (Fig. 3D). To determine whether Spp1 induces MMP9 expression *in vivo*, we assessed MMP9 protein levels in whole muscle lysates from *Spp1*^{-/-mdxB10} and *Spp1*^{+/+mdxB10} muscles. MMP9 was significantly reduced in the absence of Spp1 (Fig. 3E and F) supporting the hypothesis that decreased extracellular processing of TGF β is due to reduction in MMP9 in Spp1-deficient muscles. A schematic of this hypothesis is shown in Figure 3G.

Inhibition of MMP9 reduces TGF β extracellular processing and collagen I expression

Active TGF β is secreted in a complex with its inactive domain (referred to as the small latent complex [SLC]). SLC binds to latent TGF β binding protein (LTBP) to form a large latent complex (LLC) (Fig. 4A). Among the members of the LTBP family, LTBP4 is of special interest because it is preferentially expressed in skeletal and cardiac muscles and it is a known modifier of the dystrophic phenotype (3, 5). Both LTBPs and SLC can be cleaved by several extracellular proteinases including MMP9 (25).

To test whether MMP9 is involved in TGF β processing, we incubated primary skeletal muscle fibroblasts with *Spp1*^{+/+mdx} CM in the presence or absence of a MMP9-specific inhibitor. Inhibition of MMP9 activity led to accumulation of the SLC in CM suggesting that MMP9 regulates extracellular TGF β activity by cleaving the SLC (Fig. 4B). Moreover, as shown in Figure 4C, inhibition of MMP9 activity reduced collagen I

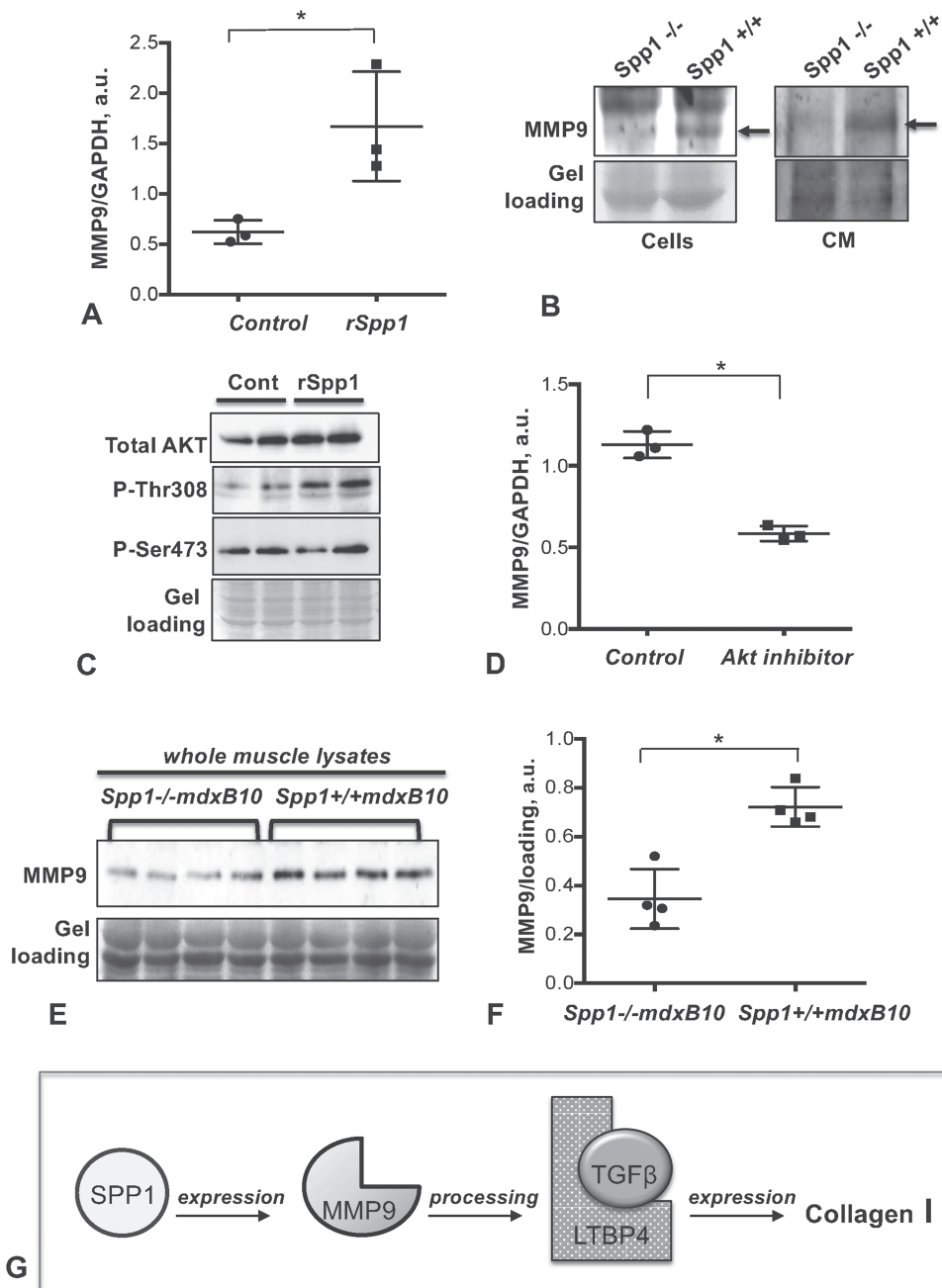


Figure 3. Spp1 regulates expression of Mmp9 that contributes to the proteolytic processing of TGF β . (A) rSpp1 upregulates expression of Mmp9 in *Spp1*^{-/-}mdxB10 fibroblasts as assessed by quantitative RT-PCR. (B) Western blot analysis showed increased Mmp9 inside the cells and in concentrated CM from *Spp1*^{+/+}mdxB10 fibroblasts compared to *Spp1*^{-/-}mdxB10 fibroblasts. (C) rSpp1 activates Akt signaling pathway as revealed by increased phosphorylation of Thr308 (D). Expression of Mmp9 gene was significantly decreased by incubation with Akt inhibitor (HY-15431). (E) Western blot analysis of total protein lysates from *Spp1*^{-/-}mdxB10 and *Spp1*^{+/+}mdxB10 mice (n=4 of each genotype) showed that Spp1 regulates Mmp9 expression in vivo; quantitative analysis of the blot is shown in (F). (G) Schematic representation of the hypothesis that Spp1 regulates expression of Mmp9, which contributes to proteolytic processing of TGF β . Asterisk indicates statistical significance of $P < 0.05$; AU, arbitrary units.

expression in a dose-dependent manner. Thus, these data suggest that MMP9-mediated processing and activation of TGF β could underlie promotion of the fibrotic process in dystrophic muscles.

To determine whether these relationships exist in vivo and to confirm that MMP9 plays a role in controlling latent TGF β processing, we compared the levels of active TGF β in double mutant *Mmp9*^{-/-}mdxB10 and *Mmp9*^{+/+}mdxB10 muscles (Fig. 4D). These

mice have been previously described (23), and *mdxB10* lacking MMP9 were shown to have a milder dystrophic phenotype, likely due to decreased fibrosis and improved muscle regeneration (26). In agreement with these previously published data, we also showed that the levels of active TGF β were significantly lower in *Mmp9*^{-/-}mdxB10 compared to *Mmp9*^{+/+}mdxB10 muscles (Fig. 4E and F), suggesting that MMP9 activity correlates with TGF β processing in vivo. Taken together, our data suggest that the

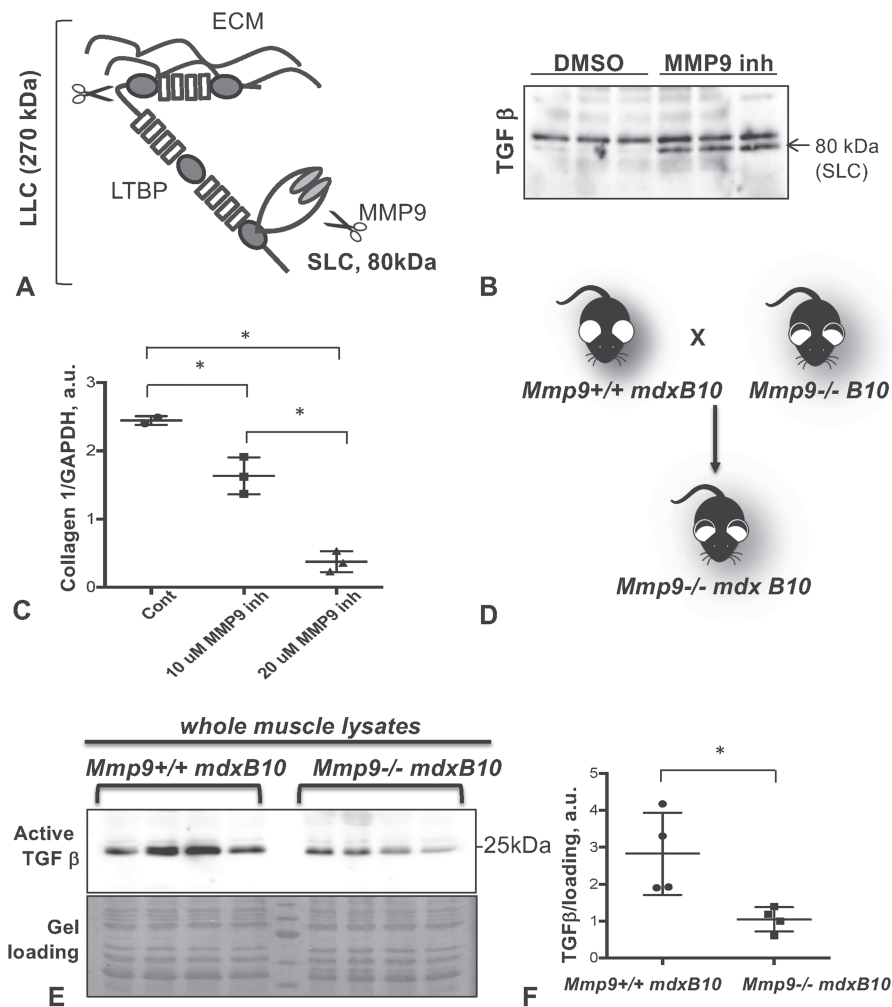


Figure 4. Mmp9 contributes to proteolytic processing of TGFβ precursor. (A) Schematic structure of SLC and LLC of TGFβ precursor that can both be cleaved by Mmp9. (B) CM of fibroblasts incubated with or without Mmp9 inhibitor shown in triplicate (Enzo Life Sciences) were probed with anti-TGFβ antibody. Arrow indicates 80 kDa SLC, which is accumulated in CM containing Mmp9 inhibitor. (C) Inhibition of Mmp9 activity was associated with a dose-dependent decrease in collagen I expression. (D) Schematic of a genetic cross to produce *Mmp9*^{-/-}*mdxB10* double mutant mice. (E) Western blot analysis of total protein lysates from *Mmp9*^{-/-}*mdxB10* and *Mmp9*^{+/+}*mdxB10* mice (n = 4 of each genotype) showed decreased active form of TGFβ (25 kDa) in *Mmp9*-deficient mice. Quantitative analysis of the western blot is shown in (F). Asterisk indicates statistical significance of $P < 0.05$; AU, arbitrary units.

effect of *Spp1* on fibrosis is mediated through induction of MMP9 and processing of latent TGFβ.

Effect of *Spp1* ablation on fibrosis is diminished in the context of high TGFβ activity

To test the proposed model that *Spp1* acts upstream of MMP9 to modulate TGFβ signaling and fibrosis in dystrophic muscles, we examined the effect of *Spp1* ablation on the *mdxD2* phenotype. Muscles of *mdxD2* mice have poorly regulated TGFβ activity due to an in-frame deletion in the *LTBP4* gene that is not present in the *B10* background (3). This SNP generates an allelic variant of *LTBP4* that is more susceptible to proteolysis, leading to increased release of TGFβ from the LLC. This model is ideal for addressing mechanisms of fibrosis in a dystrophic context, since *mdxD2* mice are more severely fibrotic than *mdxB10* mice (27). Our prior studies showed that genetic ablation of *Spp1* on the *mdxB10* background decreases fibrosis, as measured by expression of collagen I, and hydroxypro-

line assay (19). If TGFβ acts downstream of *Spp1* to control expression of fibrotic genes, then one would expect little or no effect of *Spp1* ablation in the context of unregulated TGFβ signaling.

To test this hypothesis, we generated *Spp1*^{-/-} mice on the *mdxD2* background (Fig. 5A) and assessed levels of active TGFβ and fibrosis. Unlike on the *B10* background, the levels of active TGFβ were not different between *mdxD2* and *Spp1*^{-/-}*mdxD2* mice (Fig. 5B and C). Moreover, lack of *Spp1* in *mdxD2* mice had no effect on hydroxyproline content nor on collagen I gene expression (Fig. 5D and E). Furthermore, immunostaining collagen I on cross sections did not reveal any significant differences between *mdxD2* and *Spp1*^{-/-}*mdxD2* muscles (Fig. 5F). Thus, in the *mdxB10* background, ablation of *Spp1* significantly decreased active TGFβ and improved fibrosis; in contrast, lack of *Spp1* in the context of unregulated TGFβ activity (as in the *mdxD2* background) had only a modest effect on the level of fibrosis. These data indicate that *Spp1* acts upstream of the TGFβ pathway in control of pro-fibrotic gene expression to impact ECM deposition in dystrophic muscles.

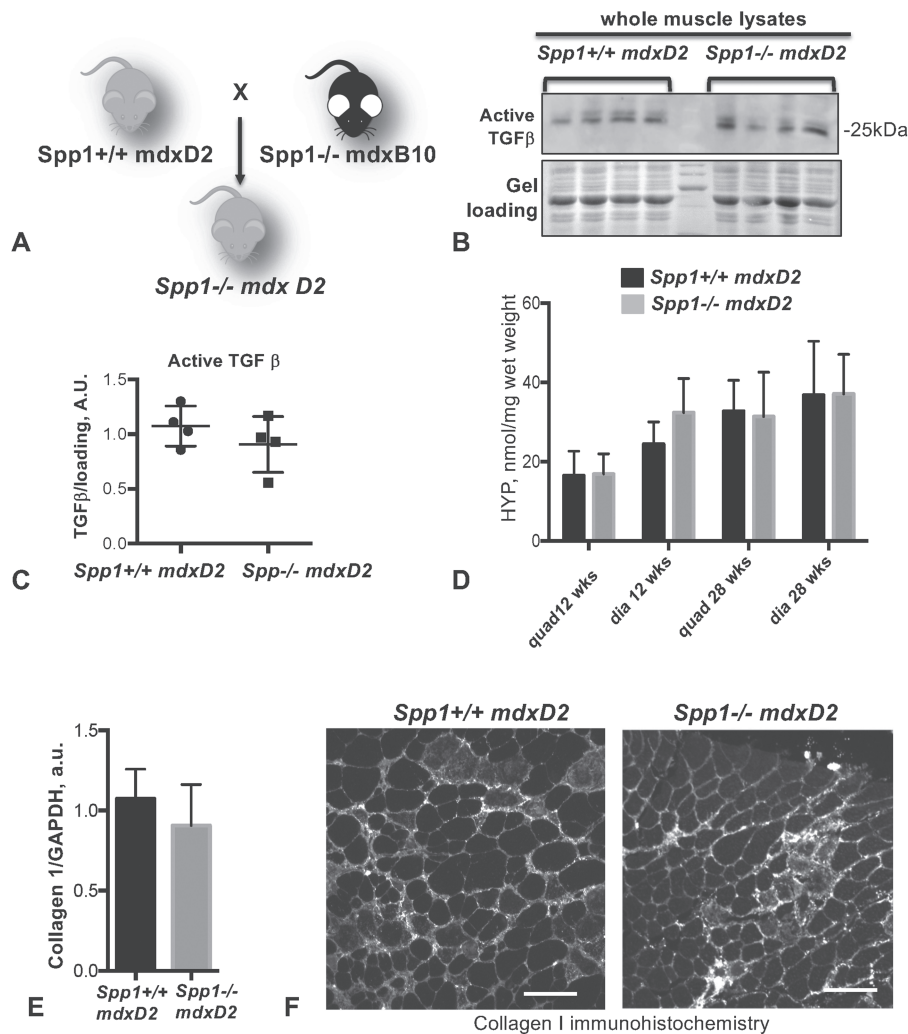


Figure 5. Effect of *Spp1* ablation in *mdxD2* mice. (A) Schematic of a genetic cross to produce *Spp1*^{-/-}*mdxD2* mice. (B) Western blot analysis of the total protein lysates from *Spp1*^{-/-}*mdxD2* mice and *Spp1*^{+/+}*mdxD2* mice ($n = 4$ of each genotype). The active form of TGF β (25 kDa) was not different between the two genotypes. Quantitative analysis of the western blot is shown in (C). Asterisk indicates statistical significance of $P < 0.05$; AU, arbitrary units. (D) Analysis of total collagen content in 12- and 28-week-old *Spp1*^{-/-}*mdxD2* mice and *Spp1*^{+/+}*mdxD2* mice using hydroxyproline (HYP) assay. (E) RT-PCR analysis of collagen I expression did not show a difference between *Spp1*^{-/-}*mdxD2* and *Spp1*^{+/+}*mdxD2* muscles. (F) Immunofluorescent staining using anti-collagen I antibody. No significant differences were observed between *Spp1*^{+/+}*mdxD2* and *Spp1*^{-/-}*mdxD2* muscles.

Postnatal pharmacological inhibition of *Spp1* decreases active TGF β and improves fibrosis and muscle function in *mdxB10* mice

To test whether postnatal reductions of *Spp1* levels can improve fibrosis and muscle function in *mdxB10* mice, we used a *Spp1*-inhibiting compound (PTC-549) that was identified by PTC Therapeutics in a high throughput screen utilizing their GEMSTM discovery platform (28). PTC-549 efficiently reduced levels of *Spp1* in both human and mouse dystrophin-deficient fibroblasts (Fig. 6A and B). Compound PTC-549 was well tolerated by mice up to 100 mg/kg, and oral administration was determined as an optimal route of delivery. Ten days of treatment of *mdxB10* mice with 10 mg/kg PTC-549 resulted in a 30% reduction of *Spp1* in quadriceps compared to vehicle-treated mice (Fig. 6C). Using this regimen, *mdx* mice were treated for 6 months and evaluated for muscle strength by wire mesh test (after 4 and 23 weeks of treatment) and then assayed for markers of fibrosis at the end of the treatment. As shown in Figures 6D and F and Fig. 7, mice treated with PTC-549 showed a significant reduction in

active TGF β levels and collagen content (assayed by hydroxyproline) after long-term treatment. Moreover, a significant improvement in muscle strength was observed after 4 weeks and up to 23 weeks of treatment. (Fig. 6G). Immunohistochemical examination of the diaphragm muscles from mice treated with PTC-549 for 6 months revealed the presence of clusters of regenerative fibers, positive for developmental myosin heavy chain (devMyHC). These data suggest that postnatal inhibition of *Spp1* ameliorates fibrosis and improves muscle function in a mouse model of DMD.

Discussion

Excess ECM deposition or fibrosis develops in tissues undergoing chronic damage and repair, such as in the case of DMD, in which muscles sustain repeated contraction-induced sarcolemmal injury followed by muscle stem cell-mediated repair. Inflammatory cells play a necessary and important role in the process of muscle repair; thus, it is critical that signals derived

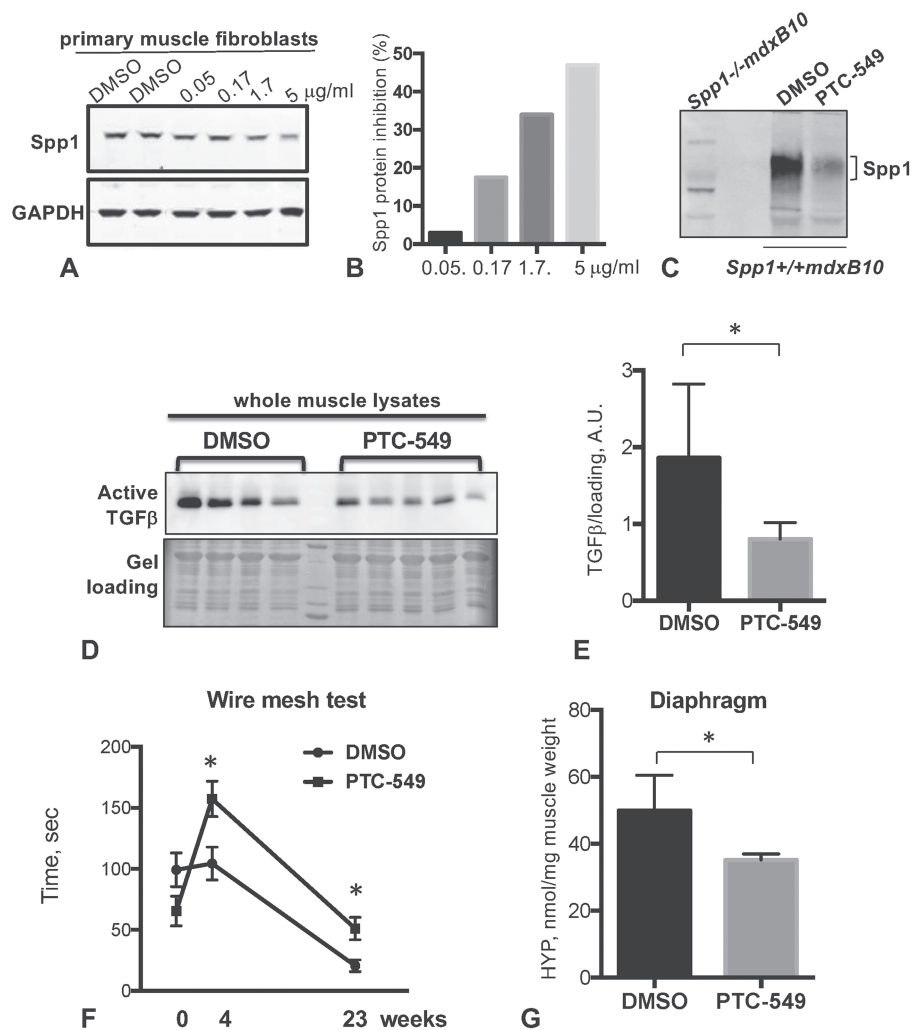


Figure 6. Effect of postnatal pharmacological inhibition of Spp1 in *mdxB10* mice. (A) Western blot of Spp1 levels in *mdxB10* primary fibroblasts that were incubated with increasing concentration of PTC-549 for 72 h. Blot was probed with anti-Spp1 antibodies (upper panel). Lower panel shows GAPDH, which was used as a loading control. (B) Quantitative analysis of the western blot shown in (A). Bars represent the percent of Spp1 inhibition relative to DMSO control. (C) Western blot of whole muscle lysates from DMSO- or PTC-549-treated mice ($n = 5$ per group in pooled samples) probed with anti-Spp1 antibody. First lane shows muscle lysate lacking Spp1 to ensure antibody specificity. (D) Western blot analysis of total protein lysates from *mdxB10* mice treated with PTC-549 or DMSO for 6 months ($n = 4$ per group). The data showed decreases in the active form of TGF β (25 kDa) in PTC-54-treated mice. Quantitative analysis of the western blot is shown in (E). (F) Wire mesh test showed that time on the wire was significantly higher for mice treated with PTC-549 at 4 weeks of treatment and up to 23 weeks ($n = 8$ and 9 mice per group). (G) Analysis of total collagen content after 24 weeks of treatment with PTC-549 or DMSO using hydroxyproline (HYP) assay. Asterisk indicates statistical significance of $P < 0.05$; AU, arbitrary units.

from these cells appear in the lesion with the correct order and timing to facilitate removal of damaged tissue and to facilitate regeneration (29). Since dystrophic lesions arise randomly and asynchronously along a muscle fiber, the carefully orchestrated repair process is ultimately undermined, resulting in accumulation of TGF β , incomplete repair and accretion of excess connective tissue (i.e. fibrosis). It has long been assumed that TGF β in the tissue derives from inflammatory cells that enter the muscle; however, these studies indicate that fibroblasts are not only a fundamental target but also a pivotal source of TGF β that contributes to muscle fibrosis. Our studies go further to demonstrate a substantial role for Spp1 on fibroblasts as a participant in this process (30).

Our prior studies revealed an overall reduction in TGF β levels and fibrosis in muscles of genetically modified mice that were globally lacking Spp1 (10,19). Subsequent analysis of the effects of Spp1 ablation on the immune infiltrate failed to reveal the cell source responsible for the reduced TGF β (9). The work presented

here strongly supports the concept that reduced fibrosis on Spp1 ablation is due to a change in processing of fibroblast-derived TGF β , increased AKT signaling and MMP9 expression in dystrophic fibroblasts. We first demonstrated a relationship between fibroblast-derived Spp1 and MMP9 in cultures treated with rSpp1 and AKT inhibitors but then confirmed the *in vivo* relevance by assessing MMP9 levels and active TGF β in Spp1 $^{-/-}$ *mdxB10* mice. This analysis revealed a significant reduction in MMP9 levels in the setting of Spp1 ablation *in vivo*. While a relationship between Spp1 and MMP9 has been previously reported (31), our studies identified muscle fibroblasts as the site of action of this pathway. Furthermore, we and others (23,32) observed a dramatic reduction in the concentration of processed TGF β in *Mmp9* $^{-/-}$ *mdxB10* mice, consistent with the hypothesis that MMP9 plays a role in promotion of TGF β activation. *Mmp9* is highly elevated in dystrophic muscles and its genetic ablation improves the dystrophic phenotype (26). Upregulation of highly similar MMP2 in dystrophic muscles has also been demonstrated

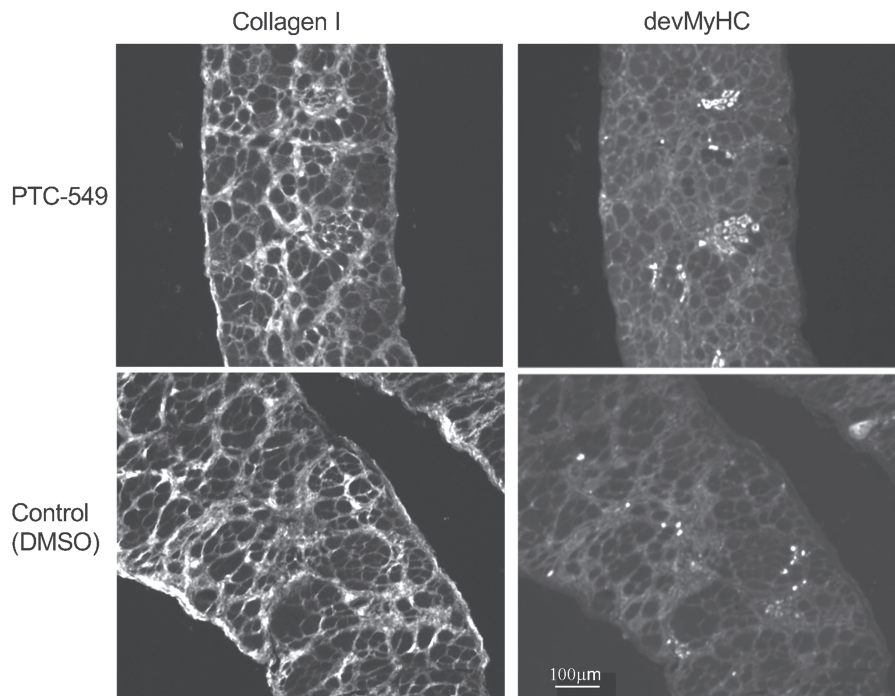


Figure 7. Immunohistochemical staining of diaphragm isolated from mice treated with PTC-549 (upper panel) or DMSO (lower panel). Frozen sections of diaphragm from mice treated with PTC-549 for 6 months stained for collagen I or developmental myosin heavy chain (a marker of regenerating fibers). All images were taken at the same magnification and the same exposure.

in mdx mice and DMD patients; however, genetic ablation of MMP2 in mdx mice had a deleterious effect on disease progression due to reduced angiogenesis and impaired muscle regeneration (33). Both MMP2 and MMP9 have been shown to cleave latent TGF β (34); however, despite their similarities, MMP2 and MMP9 play very distinct roles in dystrophic muscles and we have demonstrated a specific role for MMP9, independent of MMP2, in promoting TGF β processing in this context.

The identification of genetic modifiers of DMD that affect active TGF β levels (such as LTBP4 and Spp1) and the abundant data linking TGF β to fibrosis has lent strong support for the idea that excessive TGF β activity and fibrosis promote DMD disease progression (3,5,7); however, the mechanism has not been entirely clear. One explanation is that the excess ECM deposition that occurs in fibrosis presents a physical barrier to muscle regeneration. Another is that increased matrix stiffness that is associated with fibrosis causes cellular reprogramming of myoblasts (35), leading to lost regenerative potential. Furthermore, it is well known that TGF β can directly inhibit regeneration by blocking terminal myogenic differentiation (36); thus, TGF β can also directly interfere with regenerative potential even in the absence of excess ECM, and these effects of TGF β may be additive.

Studies in different muscular dystrophy patients have provided mixed results regarding a Spp1 modifier effect (37). For example, it has been reported that Spp1 does not act as a disease modulator in congenital muscular dystrophy type 1A indicating that Spp1's ability to modulate dystrophic phenotype may be context dependent (38). Moreover, Spp1 has been shown to be post-translationally modified by phosphorylation, glycosylation, sulfation, transglutamination and proteolytic cleavage (39) leading to numerous forms, each with the capacity to act on a wide range of receptors. MMPs have been shown to cleave Spp1 to expose an RGD peptide that enhances Spp1's ability

to bind to integrins to affect cellular behavior (13,14). Thus, different post-translationally modified forms of Spp1 can combine with a large number of potential receptors to regulate a diverse number of physiological and pathological processes. As an example, it has been shown that Spp1 affects sarcolemmal repair through increased TGF β signaling and induction of *Slug* and *Snail* occupancy on the annexin promoter, leading to inhibition of annexin expression (19). Annexins are important repair-promoting proteins, and thus, their inhibition leads to reduced efficiency of membrane repair in myofibers after induced injury. This example highlights a specific interaction between Spp1 and myofibers.

Taken together with our previous work, these studies suggest that Spp1's effects on the dystrophic process are multi-faceted. On the *mdxB10* background, loss of *Spp1* reduces TGF β and fibrosis and increases muscle growth and regeneration through its actions on fibroblasts and macrophages, respectively. While the current work reveals a direct relationship between Spp1 and promotion of fibrosis by fibroblasts, our prior data provide support for Spp1's modulation of macrophage polarization in mdx muscles (9,10). M2c macrophages accumulate in dystrophic muscles and secrete growth factors essential for muscle repair. Ablation of *Spp1* shifted macrophage polarization toward the M2c pro-regenerative phenotype and led to an increase in their secretion of IGF1 and LIF. Thus, Spp1 affects muscle regeneration and growth through effects on macrophage polarization toward M2c and increased levels of insulin like growth factor (IGF) and leukemia inhibitory factor (LIF) (9), while it impacts fibrosis through its effects on fibroblasts and increases in MMP9 that promotes TGF β processing.

Our studies reveal two distinct benefits of Spp1 ablation in dystrophic muscles: 1) reduced fibrosis through decrease of both MMP9 and TGF β activation in fibroblasts and 2) promotion of muscle growth/regeneration through skewed macrophage polar-

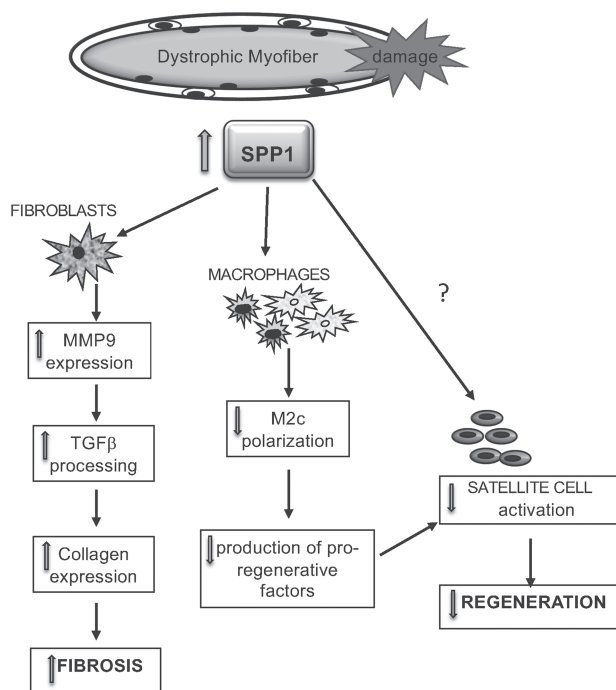


Figure 8. Schematic of *Spp1* effects on different cell populations in dystrophic muscle. *Spp1* is elevated in dystrophic muscles. *Spp1* causes macrophages to polarize away from M2c and reduce their growth factor secretion of IGF1 and LIF. *Spp1* also acts on fibroblasts to induce MMP9 expression, which leads to processing of TGF β .

ization that leads to their increased growth factor secretion (Fig. 8). Whether or not *Spp1* directly affects muscle precursor cells and/or influences myogenic differentiation in dystrophic muscle remains to be determined. Recent studies using a whole muscle autograft model of muscle injury concluded that *Spp1* derived from both muscle and non-muscle sources (primarily, inflammatory cells) is equally important for normal muscle recovery after acute injury; however, because these studies used whole muscle grafts, neither the essential source(s) of *Spp1* nor the mechanisms of *Spp1*'s action were determined (40). *Spp1*'s effects may depend on its cellular origin as well as the genetic context in which it acts (acute injury or otherwise healthy muscle vs. chronic injury associated with muscular dystrophy).

Since our data suggest that genetic ablation of *Spp1* can ameliorate DMD disease progression in *mdxB10* mice, we tested whether *in vivo* targeting of *Spp1* is beneficial to the dystrophic phenotype, using a new compound (PTC-549) that targets *Spp1*. Similar to *Spp1*^{-/-}*mdxB10* mice (9,10), PTC-549-treated *mdxB10* mice showed significant improvements in muscle strength and fibrosis, thus providing proof of concept that post-natal ablation of *Spp1* is beneficial for the dystrophic phenotype. This work opens the possibility of using pharmacological agents that target *Spp1* to improve or delay fibrosis in DMD, which may also be beneficial when used in combination with gene or cell therapies.

Materials and Methods

Mice

Spp1 and *Mmp9* knockout mice were obtained from Jackson laboratory. *Spp1*^{-/-}*mdxB10* mice were generated and described previously (10). To generate *Spp1*^{-/-}*mdxD2* mice, *Spp1*^{-/-}*mdxB10* mice were bred with *mdxD2* mice (The Jackson Laboratory, Bar

Harbor, Maine) for over 5 generations. The following primers were used for genotyping of D2 background, forward 5' AACGGC-TACCCAAAGCTTCA and reverse 5' AGGCTTCTGCTACTGCTC. A 36 bp deletion/insertion in *Ltbp4* gene was detected by genotyping PCR (3). *Mmp9*^{-/-}*mdxB10* mice were generated by breeding *Mmp9*^{-/-} mice and *mdxB10* mice. Previously published protocols were used for genotyping of *mdx* allele (41,42) and for *Mmp9*^{-/-} (The Jackson Laboratory).

For PTC-549 studies, *Spp1*^{-/-}*mdxB10* mice were housed individually during a course of treatment. In addition to regular pellets, each mouse received 1 g of peanut butter containing PTC-549 in Dimethyl Sulfoxide (DMSO) (10 mg/kg daily) or equal volume of DMSO (control group). All animals were handled and bred according to guidelines stipulated by the Animal Research Committee at UCLA.

Cell culture

Primary skeletal muscle fibroblasts were isolated from all hindlimb muscles of three 10- to 13-day-old *Spp1*^{-/-}*mdxB10* mice, washed with sterile PBS and minced in 1:1 mixture of 1600 U/ml collagenase type 2 and 1.5 mg/mg dispase (Worthington Biochemical Corp., Lakewood, New Jersey) muscle homogenate was incubated with collagenase for 30 min at 37°C with slow agitation. Once the tissue was digested, muscle homogenate was diluted with sterile PBS and passed through a 70- μ m cell strainer. Cells were pelleted by centrifugation at 900g for 5 min and resuspended in growth medium (F10 Ham [Sigma], supplemented with 1% penicillin/streptomycin and 20% fetal bovine serum (FBS)). During next 2–3 passages, cells were pre-plated for 30 min to allow highly adhesive fibroblasts to attach to the plate. After 30 min, the medium with unattached cells was removed and fresh growth medium was added. To collect CM, confluent cells were incubated with serum free F10 Ham medium for 24 hours. Vivaspin 6 tubes (GE Healthcare, Pittsburgh, Pennsylvania) were used to concentrate CM for immunoprecipitation of *Spp1* and for Western blotting. To inhibit MMP9 activity, cells were incubated with 10 μ M or 20 μ M MMP9 inhibitor I (Enzo Life Sciences, Farmingdale, NY) for 24 hours. To inhibit TGF β , cells were incubated with 10 μ M of SB431542 (Tocris) for 24 hours. To inhibit AKT, cells were incubated with 10 μ M HY-15431 MedChemExpress (MCE) for 24 hours. For PTC studies, *mdxB10* mouse primary fibroblasts were incubated with increasing concentrations of PTC-549 (0.05 to 5 μ g/ml) for 72 hours.

Immunoprecipitation of *Spp1* from concentrated CM

BrCN-Sepharose (Sigma-Aldrich, Saint Louis, Missouri) was activated by incubation with 1 mM HCl for 2 hours at 4°C. After centrifugation at 1000g for 5 min, activated beads were washed once in coupling buffer (100 mM NaHCO₃, 500 mM NaCl, pH 8.3), resuspended in coupling buffer with goat anti-mouse *Spp1* antibody (R&D Systems, Minneapolis, Minnesota) at 50 μ g/ml and incubated overnight at 4°C. Beads were washed with coupling buffer for 30 min at room temperature and incubated with quenching buffer (100 mM Tris-HCl, pH 8.0) for 2 hours at room temperature. Concentrated CM were pre-cleaned by incubation with BrCN-Sepharose and added to the beads with linked antibodies for overnight incubation at 4°C. After that, beads were washed three times in PBS with 0.1% Tween 20. To eluate bound proteins, beads were incubated for 5 min with elution buffer (100 mM glycine, 2.5 pH, 500 mM NaCl). Eluates were neutralized by 1:10 volume of 1 M Tris pH 8.0 and analyzed by Western blotting. Concentration of immunoprecipitated *Spp1* was determined by comparing with a range of known amounts of recombinant mouse *Spp1* (R&D Systems) loaded on the same gel.

Western blotting

Cell pellets were resuspended in reducing sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% Sodium Dodecyl Sulfate (SDS), and 100 mM β -mercaptoethanol) supplemented with protease and phosphatase inhibitors (Sigma, St Louis, Missouri). DNA was shared by passing through 27G needle 10 times. The same reducing sample buffer (30 V/muscle weight) was used to homogenize whole muscle. After electrophoresis, proteins were transferred to nitrocellulose membrane. The following primary antibodies were used for Western blot analysis: anti-TGF β (R&D), anti-MMP9 (Cell Signaling, Danvers, Massachusetts). Imaging was performed using c300 imager (Azure Biosystems, Dublin, California), and Western blot quantification was done using Image J software.

Real-time PCR

cDNA was generated using iScript Reverse Transcriptase Supermix (Bio-Rad, Hercules, CA) and was used for real-time PCR (RT-PCR) with iTaq Universal SYBR Green Supermix (Bio-Rad) according to manufacturer's instructions. All RT-PCR reactions were run in CFX Connect Real-Time PCR System (Bio-Rad). Primers for RT-PCR were selected to span intron-exon junctions (when possible) and were first tested in regular PCR amplification to ensure the production of a single band in each case. The following primer pairs were used:

GAPDH Frw 5' tccaccacctgtgtctgta and Rev 5' gacttcaacagcaactccac.

collagen I Frw 5' gtcgcttcacctacagcac and Rev 5' caatgtccaaggagccac.

MMP9 Frw 5' gatccccagagcgtcattc and Rev 5' ccactgtttcacctctttt.

TGF β 1 Frw 5' gggaagcagtgcccgaacc and Rev 5' tgggggtcagcagcggta.

Immunohistochemistry

Frozen quadriceps cross-sections were fixed for 10 min with 4% paraformaldehyde and stained with rabbit anti-collagen I antibody (Cedarlane Laboratories, Burlington, Canada) followed by secondary anti-rabbit FITC antibody (Vector). Slides were analyzed using AxioVision Software from Zeiss.

Supplementary Material

Supplementary Material is available at HMG online.

Conflicts of Interest statement

E.W. is an employee of PTC therapeutics, which provided PTC-549 compound for these studies. The other authors have no conflicts of interest.

Funding

National Institute of Arthritis and Musculoskeletal and Skin Diseases for a Wellstone Cooperative Muscular Dystrophy Center [U54AR052646]; P30 Muscular Dystrophy Core Center [NIAMS-P30AR057230-01 to M.J.S.]; National Institutes of Health grants [R01 AR046911 to M.J.S., R01 HL140938 to E.M.M.]; Parent Project Muscular Dystrophy (M.J.S.); Muscular Dystrophy Association [MDA Development Grant No. 479350 to M.J.S. and M.Q.].

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