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ORIGINAL PAPER

PDGFRα signalling promotes fibrogenic responses in collagen-producing cells in Duchenne muscular dystrophy

Nicholas Ieronimakis,[1] Aislinn Hays,[2] Amalthiya Prasad,[1] Kajohnkiart Janebodin,[3] Jeremy S Duffield[1,4,5] and Morayma Reyes[2,6,*]

[1] *Department of Pathology, School of Medicine, University of Washington, WA, USA*
[2] *Department of Pathology, Albert Einstein College of Medicine, NY, USA*
[3] *Department of Anatomy, School of Dentistry, Mahidol University, Thailand*
[4] *Department of Medicine, School of Medicine, University of Washington, WA, USA*
[5] *Discovery Research, Biogen Inc, Cambridge, MA, USA*
[6] *Montefiore Medical Center, Bronx, NY, USA*

**Correspondence to: M Reyes, Department of Pathology, Albert Einstein College of Medicine, 1300 Morris Park Ave, Bronx, NY 10461, USA. E-mail: mreyesgi@montefiore.org*

Abstract

Fibrosis is a characteristic of Duchenne muscular dystrophy (DMD), yet the cellular and molecular mechanisms responsible for DMD fibrosis are poorly understood. Utilizing the *Collagen1a1-GFP* transgene to identify cells producing Collagen-I matrix in wild-type mice exposed to toxic injury or those mutated at the dystrophin gene locus (*mdx*) as a model of DMD, we studied mechanisms of skeletal muscle injury/repair and fibrosis. PDGFRα is restricted to Sca1+, CD45− mesenchymal progenitors. Fate-mapping experiments using inducible CreER/LoxP somatic recombination indicate that these progenitors expand in injury or DMD to become PDGFRα+, Col1a1-GFP+ matrix-forming fibroblasts, whereas muscle fibres do not become fibroblasts but are an important source of the PDGFRα ligand, PDGF-AA. While in toxin injury/repair of muscle PDGFRα, signalling is transiently up-regulated during the regenerative phase in the DMD model and in human DMD it is chronically overactivated. Conditional expression of the constitutively active PDGFRα *D842V* mutation in Collagen-I+ fibroblasts, during injury/repair, hindered the repair phase and instead promoted fibrosis. In DMD, treatment of *mdx* mice with crenolanib, a highly selective PDGFRα/β tyrosine kinase inhibitor, reduced fibrosis, improved muscle strength, and was associated with decreased activity of Src, a downstream effector of PDGFRα signalling. These observations are consistent with a model in which PDGFRα activation of mesenchymal progenitors normally regulates repair of the injured muscle, but in DMD persistent and excessive activation of this pathway directly drives fibrosis and hinders repair. The PDGFRα pathway is a potential new target for treatment of progressive DMD.

Keywords: PDGFRα signalling; fibrosis; collagen-producing cells; Duchenne muscular dystrophy; skeletal muscle

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Introduction

Duchenne muscular dystrophy (DMD) is a fatal disease primarily affecting skeletal and cardiac muscles. There are no approved treatments that halt or reverse the course of the disease. As the disease progresses, connective tissue accumulates in degenerating cardiac and skeletal muscles, a process commonly referred to as fibrosis [1]. Fibrosis is the most prominent pathological feature, and a reliable determinant of disease progression, yet the cellular and molecular milieu that governs fibrosis in DMD remains largely uncharacterized [1–3]. Skeletal muscle mesenchymal progenitors capable of differentiating into resident fibroblasts and adipocytes have been identified (also referred to as FAPs) [4–6]. These mesenchymal progenitors are PDGFRα+ cells that have been associated with the pathogenesis in *mdx* and DMD skeletal muscles [7,8]. Recently, these mesenchymal progenitors in the skeletal muscle and heart have been shown to express pro-fibrotic genes in response to TGF-β1 and PDGF-AA *in vitro*, implicating them as potentially important cells in the fibrogenic process [7,9].

Constitutive activation of PDGFRα has been reported to induce spontaneous systemic fibrosis in healthy mice, and pro-fibrotic cells present in many organs express PDGFRα [7,9–12]. In models of toxin injury to skeletal muscle, pro-fibrotic cells also express PDGFRα [5,7,10]. Recently, studies have highlighted a role for PDGFRα signalling in the pro-fibrotic response of not only FAPs but also myogenic, haematopoietic, and endothelial cells [13]. Imatinib, a broad spectrum tyrosine kinase inhibitor, has been reported to decrease

fibrosis in *mdx* skeletal muscles by inhibiting c-Abl and PDGFR signalling, suggesting that the PDGFR pathway may be important [6,14,15]. Taken together, these reports suggest that pro-fibrotic muscle cells can express PDGFRα and that this pathway has the potential to induce fibrosis. Despite this evidence, it remains unclear whether PDGFRα signalling directly promotes fibrosis and loss of function in DMD. In light of recent evidence indicating that multiple cell populations contribute to fibrosis [13], the proportion of PDGFRα-expressing cells that produce fibrillar collagens and promote fibrosis in muscular dystrophy needs to be carefully examined.

Skeletal muscle fibrosis in DMD is characterized by the accumulation of type I and type III collagens [16]. In cardiac muscle, fibroblasts are thought to be a heterogeneous population with various expression patterns and developmental origins, although very recent studies now suggest that the majority appear to derive from resident populations of mesenchymal cells [17–21]. In skeletal muscles, populations including FAPs and endothelial, myogenic, and haematopoietic cells have been reported to express collagens [6,13]. However, the heterogeneity and the extent of collagen expression by various muscle populations have raised more questions which necessitate greater delineation. The identification of fibrotic cells is inherently hampered by the nature of collagen synthesis, which begins intracellularly, with the expression and production of pro-collagens, and ends with the assembly of collagen fibrils in the extracellular space [22]. Therefore, collagen-producing cells are not easily distinguishable from other cell types, such as inflammatory cells, that also occupy damaged tissue and influence fibrosis in disease or injury [23].

In the present study, we used fate-mapping techniques to study and dissect the roles of PDGFRα + cells and PDGFRα signalling in disease progression in *mdx* mice which harbour a mutated dystrophin gene, drawing direct comparison with human dystrophic muscles. We show that PDGFRα + cells are the primary source of fibrillar collagen matrix synthesis in disease. By comparing acute injury followed by repair in healthy skeletal muscle with chronic disease of *mdx* muscle, we dissected the differences and similarities between the regeneration process that results in restoration of function and the maladaptive repair process that characterizes muscle dystrophy.

Materials and methods

Expanded methods and details of mouse models, primers, and antibodies are listed in the supplementary material, Supplementary materials and methods and Tables S1–S5, and elsewhere [9,24–28]. Muscle injuries were caused by a single injection of a 10 nM solution of cardiotoxin (Sigma, St Louis, MO, USA) directly into the tibialis anterior (TA; 20 µl) or quadriceps (40 µl) [29]. For inducible Cre activation, 100 µl of 20 mg/ml tamoxifen suspended in corn oil was injected intraperitoneally (i.p.) daily for five consecutive days and then allowed to resolve for a minimum of 5 days prior to cardiotoxin injury. Myography was conducted and specific isometric force was calculated as specified previously [29]. Tissue processing, histology, and staining were in accordance with published procedures [9,29,30].

Cells for molecular analysis were isolated by FACS from single cell preparations prepared by digesting a collection of limb muscles (TA, gastrocnemius, and quadriceps) with collagenase and dispase (Worthington Biochemical Corp, Lakewood, NJ, USA) [26,31]. RNA was isolated from cells or tissue as described previously [29]. Proteins were isolated from muscle tissues snap-frozen with liquid nitrogen, ground into a fine powder, weighed, suspended in RIPA buffer containing protease and phosphatase inhibitors (Thermo/Pierce, Rockford, IL, USA), and then homogenized with a Tissue-Tearor (BioSpec, Bartlesville, OK, USA). The cultured Col1a1-GFP+ cells used for *in vitro* studies were generated as reported previously [9]. Single muscle fibres were isolated and cultured as described elsewhere [32] from 4-month-old wild-type (WT) and *mdx* extensor digitorum longus (EDL) muscles.

Measurement of the percentage area staining was performed using ImageJ software (NIH) [29,33,34]. The minimum diameter of muscle fibres and densitometry were also measured by ImageJ [29]. Graphs show the mean of replicates for each condition. Student's *t*-test, or ANOVA when appropriate, was used for statistical analyses and computation of *p* values.

Results

Col1a1-GFP+ cells are responsible for connective tissue accumulation in dystrophic muscles

To study the process of fibrosis in dystrophic skeletal muscles, we utilized *mdx* mice that harbour a reporter of collagen type I production, the *Col1a1-GFP* transgene [9,35]. Col1a1-GFP+ cells are abundant in regions of pathology in *mdx* diaphragms compared with diaphragms from wild-type, healthy mice (Figure 1). Alterations in connective tissue and muscle architecture, highlighted by collagen type I staining and the presence of GFP+ cells, were prominent in diseased diaphragms and quadriceps of *mdx* mice (Figure 1A). This remodelling of the tissue architecture is characteristic of the cycles of degeneration and regeneration that occur with the progression of muscular dystrophy and presumably results in fibrosis as muscles lose their capacity to repair. Consistent with this, Col1a1-GFP+ cells are closely associated with muscle fibres positive for sarcoplasmic fibronectin, an indicator of degenerating muscle [36], and α-smooth muscle actin (αSMA), which is expressed by regenerating fibres [37] (Figure 1C, D). The cycles of damage and repair are associated with the accumulation of collagen type I and type III, the main components

of connective tissue in *mdx* muscle fibrosis [38–40]. Although expression of *Col1a1-GFP* is indicative of likely pro-collagen 1α1 production, it remained unclear if GFP+ cells were also the source of type III collagen. Therefore, we isolated Col1a1-GFP-positive and negative cells from *mdx* muscle digests by flow sorting to compare their expression of collagen mRNAs by RT-qPCR. GFP+ cells isolated from *mdx* diaphragms expressed high levels of *Col1a1* and *Col3a1*, whereas other muscle cells such as endothelial cells (CD31+, Sca1+, CD45−) and macrophages (CD45+, F4/80+) did not express these pro-collagen mRNAs (Figure 1E).

To understand the progression of fibrosis, we characterized collagen type III, which accumulates in dystrophic *mdx:Col1a1-GFP* muscles [40]. Diaphragms from *mdx* mice showed thickening of endomysial type III collagen by 4 months of age (Figure 2A). GFP+ cells were located in areas of collagen type III accumulation. In quadriceps muscles, which show less severe pathology (Figure 2B), accumulation of collagen type III was evident by 14 months of age (Figure 2B) [41]. Collagen type III was also concentrated in the perimysium surrounding large vessels and associated with intense accumulation of Col1a1-GFP+ cells (supplementary material, Figure S1A). In addition, a microfibrillar collagen, collagen type VI, which is up-regulated in DMD [42], was also present in fibrotic regions occupied by Col1a1-GFP+ cells (supplementary material, Figure S1B). The localization of both collagen type III and collagen type VI with GFP+ cells was also observed in aged hearts from the same *mdx* mice (supplementary material, Figure S2).

Col1a1-GFP matrix-forming cells are PDGFRα+, Sca1+, CD45− in dystrophic muscles and derive from MSC progenitors

To characterize the markers of Col1a1-GFP+ cells in skeletal muscle, we analysed single-cell preparations of diaphragms from *mdx* mice by flow cytometry and compared our findings with immunohistological detection in tissue sections. GFP+ cells did not express the haematopoietic or endothelial markers CD45 or CD31, respectively (Figure 3A, B) [43,44]. Evaluation of the PDGFRα cellular compartments indicates that PDGFRα+ cells expressing Col1a1-GFP are also positive for Sca1 in both wild-type and *mdx* diaphragms. In contrast, Col1a1-GFP+ cells that are PDGFRα-negative are negative for Sca1 (supplementary material, Figure S3). In *mdx* diaphragms, the majority of Col1a1-GFP+ cells are PDGFRα+/Sca1+ (Figure 3A, B) and their proportion within the non-haematopoietic population (CD45−) steadily increases with age, becoming the majority (>60%) at 20 months of age. This profiling suggests that a proportion of FAPs (PDGFRα+, Sca1+, CD45−) express Col1a1-GFP in normal muscles, while the majority adopt a fibrogenic phenotype with the progression of dystrophy. In contrast, a large proportion of Col1a1-GFP+ cells in wild-type muscle show an absence of PDGFRα/Sca1, suggesting that these are pre-committed cells in a state of inactivity.

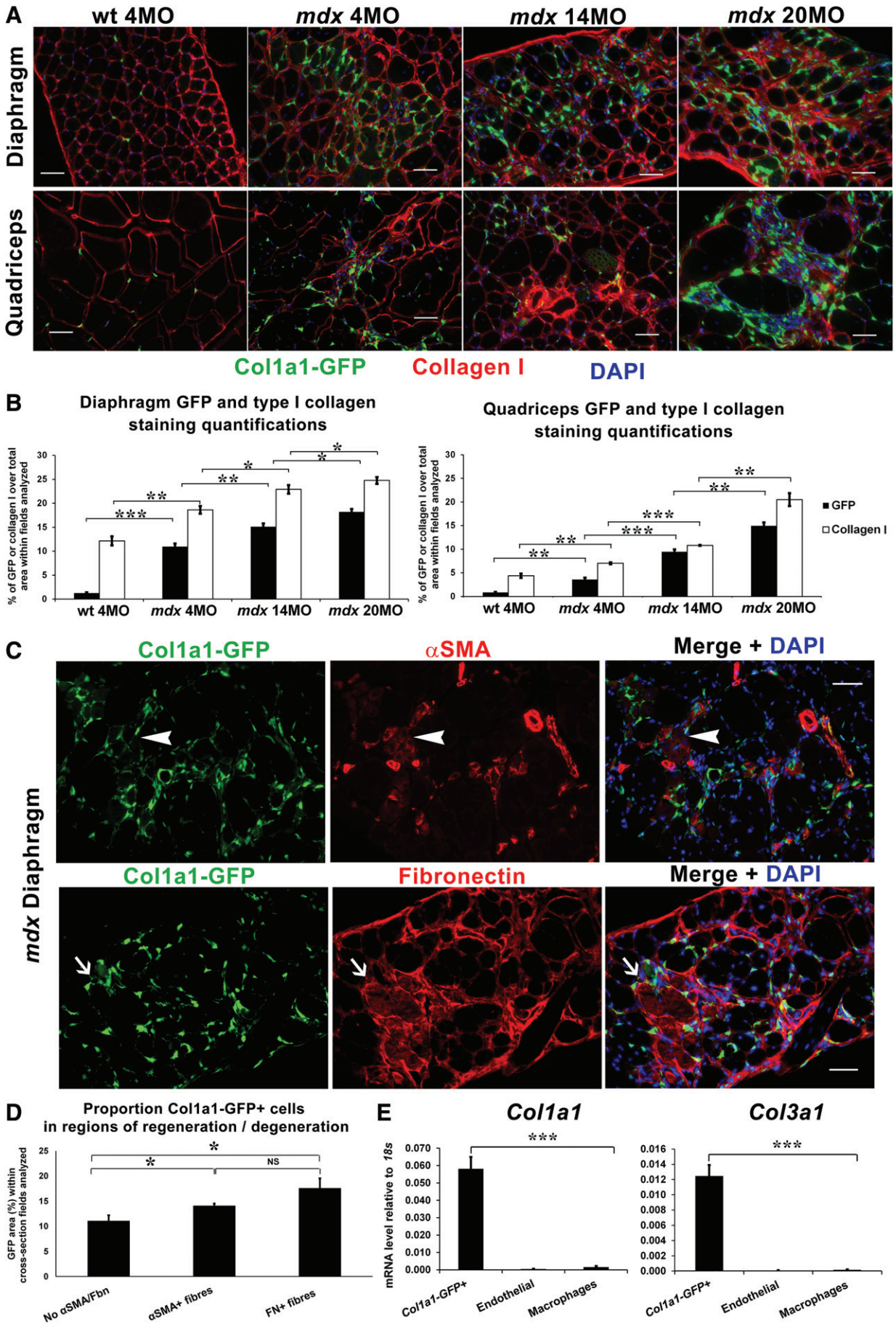
Because DMD also directly causes cardiac fibrosis, we examined cardiac Col1a1-GFP+ cells in the same way (supplementary material, Figure S4). In aged *mdx* heart muscle, more than 80% of Col1a1-GFP+ cells co-expressed PDGFRα but did not express CD31 or CD45. As previously observed, the majority of Col1a1-GFP+, PDGFRα+ cells were also Sca1+ in aged *mdx* diaphragm and heart muscles (Figure 3A and supplementary material, Figure S4) [9]. By histological analysis, Col1a1-GFP+ cells in *mdx* diaphragms co-expressed PDGFRα+, but did not co-localize with the vascular markers BS1 and NG2, which label endothelial cells and pericytes, respectively (Figure 3C) [45,46]. Therefore, the majority of Col1a1-GFP+ cells in aged *mdx* skeletal muscles share close phenotypic similarity to collagen-producing cells that have been characterized in the heart in DMD and in pressure overload cardiac failure [9,19].

To validate the exclusive expression of PDGFRα by the matrix-forming mesenchymal cells, we expressed nuclear GFP (nGFP) at the PDGFRα locus in *mdx* mice (*PDGFRa-nGFP*). Similar to Col1a1-GFP+ cells, PDGFRa-nGFP+ cells co-expressed CD34 and Sca1, but did not express CD45 and CD31 (Figure 3D). In addition, PDGFRa-nGFP+ cells were also abundant around regenerating muscle fibres and myogenic cells (Figure 3E), as well as around degenerating, necrotic muscle fibres (supplementary material, Figure S5). Quantitative analysis of RNA established that *PDGFR*α mRNA abundance was significantly elevated in skeletal and cardiac muscles in *mdx* compared with wild-type mice (Figure 3 F).

To study the response of the PDGFRα + mesenchymal progenitors to the onset and progression of dystrophy, we generated *mdx* mice carrying the *PDGFRa-Cre* allele and mT/mG loxP reporter [47,48]. In *mdx* muscle, the presence of membrane-EGFP+ cells at 6 weeks of age was similar to healthy muscle (supplementary material, Figure S6). By 8 weeks, however, there was a marked expansion of membrane-EGFP+, PDGFRα fate-mapped cells in diaphragmatic muscle. This timeframe of cell expansion is consistent with the commencement of muscular dystrophy fibrosis in *mdx* diaphragms [15,40,49]. This patterning of Col1a1-GFP+ and PDGFRa-nGFP+ or PDGFRa-Cre fate-mapped populations highlights the tight cellular localization between collagen production and PDGFRα expression during the processes of skeletal muscle regeneration and degeneration.

PDGFRα signalling by Sca1+, CD45− cells is transiently activated in acute injury–repair of skeletal muscle

Toxin injury in non-diseased muscle leads to a robust myogenic response that results in near-complete regeneration within 14 days [50]. Despite the severity of damage and alteration to the muscle architecture that occur



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with toxin-induced injury, there is no persisting fibrosis, suggesting that collagen production is in balance with the need for muscle regeneration and restoration of damaged connective tissue [50], and that resorption of accumulated matrix during regeneration is coordinated and appropriate. With recurring damage in diseased *mdx* muscles, excessive deposition and thickening of the connective tissue occur, suggesting that fibrosis results from the unbalanced accumulation of collagen during chronic cycles of muscle regeneration and unresolved degeneration. Therefore, to gain insight into the process of connective tissue restoration, we examined the relationship between Col1a1-GFP-expressing cells and myogenic factor-5-expressing myogenic cells using the *Myf5^{nlacZ}* allele following cardiotoxin (CTX) injury in wild-type mice [51].

Following CTX injection, the myogenic response in wild-type muscles peaks at day 3 post-injury [31,50]. This was evident by the prominence of Myf5$^{nlacZ/+}$ cells in CTX-injured TA muscles compared with uninjured contralateral TA muscles (Figure 4A). Col1a1-GFP+ cells were also abundant within regenerating areas of muscle and in close proximity to myogenic cells and newly regenerated muscle fibres expressing αSMA (supplementary material, Figure S7). This association indicates that Col1a1-GFP+ cells are active in parallel with myogenesis. Such activation of Col1a1-GFP+ cells and myogenic cells correlated with elevated levels of pro-collagen mRNAs at day 3 and subsequent decline by 14 days post-CTX injury (Figure 4A). Consistent with findings in *mdx* muscle, PDGFRa-nGFP cells expanded in regenerating areas and expression levels of both *Pdgfra* and *Pdgfa* mRNAs showed the same pattern of increase during the peak of myogenesis at day 3, with subsequent decline at day 14, when regeneration is concluding (Figure 4B). These findings suggest that Col1a1-GFP+ cells and PDGFRα + signalling in normal muscle repair result in restoration of the connective tissue surrounding regenerating myofibres, a process altered in muscular dystrophy.

Overactivation of PDGFRα signalling enhances fibrotic responses following muscle injury and characterizes human muscle dystrophy

PDGFR expression and activation are seen in *mdx* skeletal muscle [15], while expression and activation are also increased during muscle repair (Figure 4B). Therefore, in the context of skeletal muscle injury and repair, we tested whether constitutive activation of PDGFR signalling would result in excessive connective tissue deposition spontaneously or in response to injury. We utilized a Cre/loxP somatic cell recombination strategy to express the constitutively active mutant forms of the PDGFRs (*PDGFRa Δ842V* [10] and *PDGFRb Δ536A* [52]) in PDGFRα-expressing cells immediately prior to CTX-mediated injury, using the *PDGFRα-CreER* BAC transgene, which is activated by exposure to the oestrogen analogue tamoxifen [53]. Histological analysis showed that in *PDGFRa Δ842V* muscles, CTX injury led to excessive connective tissue reminiscent of *mdx* muscle pathology (Figure 4C, D). In contrast, injured *PDGFRb Δ536A* mutant muscles showed slightly increased fibrosis. Uninjured muscles appeared similar between each model. Excessive PDGFRα activation during the process of muscle regeneration can therefore result in fibrosis. In turn, activation of PDGFRβ may also influence the response of collagen-producing cells, but with less potency compared with PDGFRα. The elevated levels of fibrosis in mutant muscles injected with CTX correlated with smaller fibre size in injured areas (supplementary material, Figure S8), indicating that excessive PDGFRα signalling in the context of muscle injury can alter the normal processes of regeneration.

To confirm specificity of *PDGFRa-CreER* and exclude the possibility of non-specific Cre activation with CTX injury, we compared tamoxifen and vehicle-treated *PDGFRa-CreER* mice harbouring the mT/mG loxP flanked conditional reporter. In these studies, GFP+ cells, indicative of Cre-mediated recombination, were observed only in animals pretreated with tamoxifen in both injured and uninjured muscles (supplementary material, Figure S9). In contrast, vehicle-treated controls did not show detectable GFP+ cells in healthy conditions or following CTX injury (supplementary material, Figure S10).

Elevated numbers of PDGFRα + cells have been reported to co-localize with fibrosis in human DMD muscles [8]. Immunostaining of DMD muscles in our laboratory supports the presence of PDGFRα + cells in the regions of connective tissue thickening (Figure 5A). Collagen accumulation was pronounced in endomysium and perimysium surrounding vessels in DMD muscle (Figure 5B), sites where Col1a1-GFP+ cells were also concentrated in *mdx* muscles (Figure 1). Furthermore,

Figure 1. Col1a1-GFP+ collagen-producing cells accompany regeneration and degeneration in dystrophic skeletal muscles of *mdx* mice. (A, B) Immunofluorescence analysis of diaphragms and quadriceps from male wild-type and *mdx:Col1a1-GFP* mice ($N = 3$ mice per age group; 4, 14, and 20 months old) reveals that GFP+ cells and type I collagen progressively increase in dystrophic muscles. Staining for type I collagen highlights areas of muscle pathology where GFP+ cells accumulate. (C) Immunofluorescence from 4-month-old *mdx* diaphragms reveals that GFP+ cells are concentrated near regenerating α-smooth muscle actin-positive (αSMA+) muscle fibres (arrowhead) and degenerating fibres that stain positively for sarcomeric fibronectin (arrow). (D) Quantification reveals increased numbers of GFP+ cells in fields containing regenerating (αSMA+) or degeneration [fibronectin + (Fn+)] fibres versus regions within the same *mdx* diaphragm muscles negative for αSMA+ or Fn + fibres. Scale bars = 50 μm. NS = not significant; $^{*}p < 0.05$; $^{***}p < 0.0005$ by Student's *t*-test. (E) RT-qPCR analysis of *Col1a1* and *Col3a1* expressed by FACS-sorted cells from diaphragm muscles of 12-month-old *mdx:Col1a1-GFP* male mice ($N = 3$). Relative to other populations implicated in collagen synthesis, endothelial cells (Sca1+, CD31+, CD45−) and macrophages (CD45+, F4/80+), Col1a1-GFP+ cells expressed significantly higher levels of these pro-collagen mRNAs. $^{***}p < 0.0005$ by single factor ANOVA. Error bars indicate ± SEM.

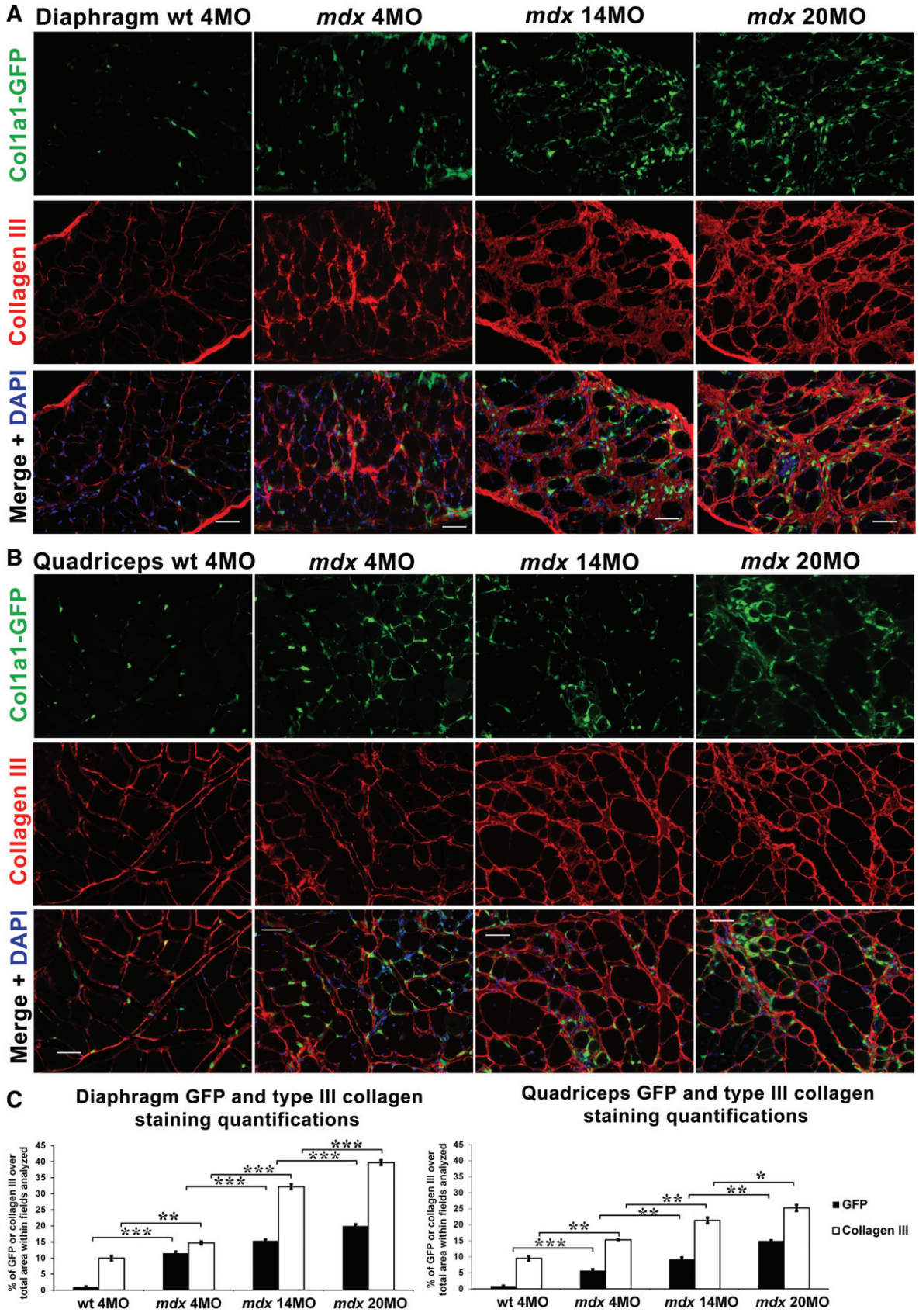


Figure 2. *Col1a1-GFP* cells accumulate in areas of fibrosis and expand with disease progression. (A) Immunodetection of type III collagen in *mdx:Col1a1-GFP* muscles shows GFP+ cells accumulated in areas of pathological matrix deposition fibrosis that occurs with age and disease progression in *mdx* diaphragms. This accumulation is observed as early as 4 months in *mdx* versus wild-type diaphragms. (B) In contrast, limb muscles are less affected in *mdx* mice. The build-up of type III collagen and co-localization with GFP+ cells were not obvious until 14 months in the quadriceps from the same *mdx* mice shown. (C) Quantification of Col1a1-GFP+ cells and type III collagen in diaphragms and quadriceps reveals progressive increase with age. Scale bars = 50 μm.

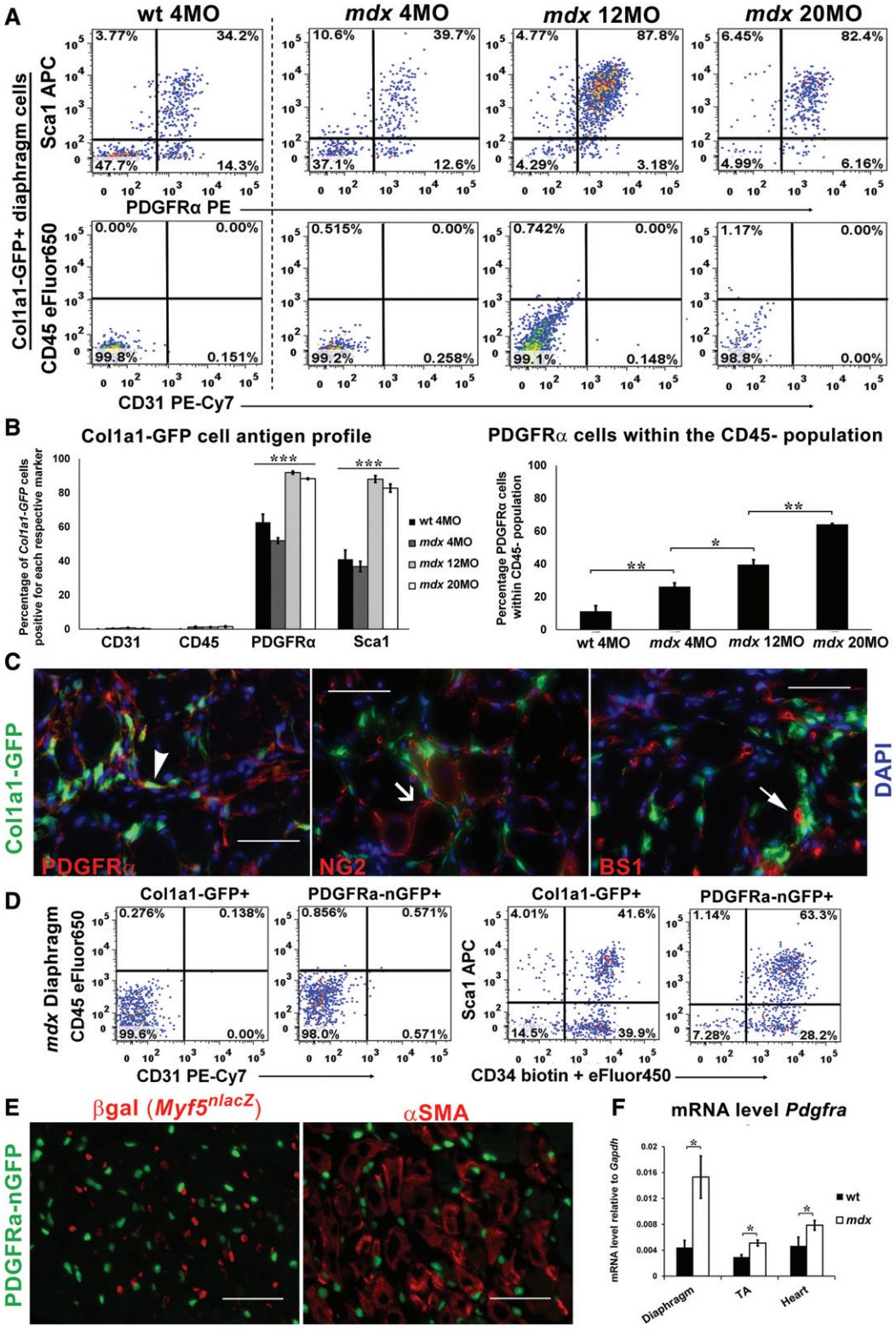
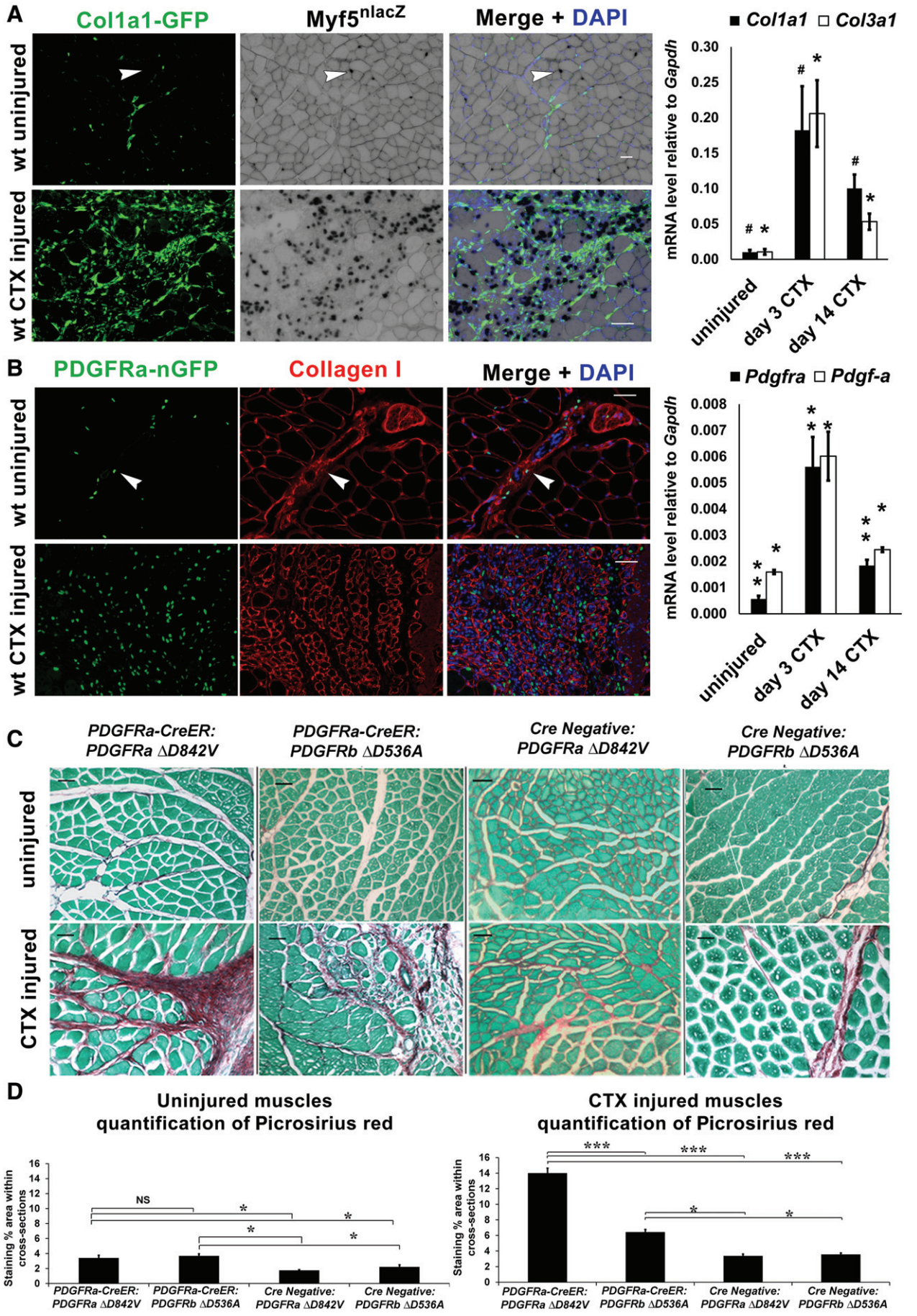


Figure 3. Col1a1-GFP+ cells express PDGFRα but not endothelial or haematopoietic markers. (A, B) FACS analysis of the Col1a1-GFP population in wild-type and *mdx* diaphragms ($N = 3$ males per age group) reveals that GFP+ cells lack CD45 and CD31, but the majority express PDGFRα and Sca1 by 12 months of age in *mdx* mice. (C) Immunofluorescence analysis of *mdx:Col1a1-GFP* diaphragms confirms that GFP+ cells co-express PDGFRα + (arrowhead), but lack vascular markers NG2 (line arrow) and BS1 (filled arrow). (D) FACS analysis comparing single-cell digests of diaphragms from *Col1a1-GFP* and *PDGFRa-nGFP* mice reveals a similar profile between GFP+ populations: negative for CD45 and CD31, but the majority co-expressing Sca1 and CD34. (E) Images showing PDGFRa-nGFP+ cells in *mdx* diaphragms concentrated in regions with regenerating fibres expressing αSMA+ as well as $Myf5^{nlacZ}$-positive myogenic cells in young *mdx:PDGFRα-nGFP:*$Myf5^{nlacZ/+}$ mice. Scale bars = 50 μm. (F) RT-qPCR analysis for *Pdgfra* indicating increased expression in *mdx* versus wild-type skeletal tibialis anterior (TA), diaphragm, and heart muscles. $N = 3$ per tissues were analysed from 12-month-old *mdx* and wild-type mice. $^*p < 0.05$. Bars show mean ± SEM.



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staining for the PDGF-AA ligand revealed increased intensity in DMD and *mdx* muscle fibres (Figure 5C, D). Co-cultures of myofibres with Col1a1-GFP+ cells provided further evidence of PDGF production and paracrine signalling to mediate fibrogenesis. In this experiment, *mdx* myofibres showed significantly greater PDGF-AA staining and co-cultured Col1a1-GFP+ cells produced a greater amount of collagen versus wild-type fibre co-cultures (supplementary material, Figure S11). These results highlight the similarities between human and mouse disease and suggest that activation of PDGFRα signalling in muscular dystrophy promotes connective tissue accumulation. Ominously, dystrophic muscle fibres may promote fibrosis in a paracrine fashion via production and secretion of the PDGF-AA ligand.

Inhibition of PDGFRα signalling ameliorates muscular dystrophy fibrosis and improves function

Crenolanib, a potent tyrosine kinase inhibitor selective for PDGFRα, is currently in phase II clinical trials for PDGFR-associated cancers [54–56]. Compared with imatinib, crenolanib has significantly greater avidity for the active form of PDGFRα, including mutant variants such as Δ*D842V* [54]. Moreover, it has no activity against VEGFR2, Src or ABL, whose inhibition has been associated with cardiac toxicities [57]. Therefore, we administered crenolanib to Col1a1-GFP+ muscle fibroblasts initially *in vitro*, to evaluate whether PDGFRα inhibition might reduce the pro-fibrotic activity of collagen-producing cells. Col1a1-GFP+ cells treated with 10 ng/ml PDGF-AA had significantly increased relative expression of both *Col1a1* and *Col3a1* (supplementary material, Figure S12A). This response to PDGF-AA was inhibited with the addition of 1 μM crenolanib. In contrast, exposure to the same concentration of PDGF-BB did not result in the up-regulation of pro-collagen transcripts. Western blot analysis confirmed the inhibitory effect of crenolanib in reducing PDGFRα phosphorylation when cells were stimulated with PDGF-AA (supplementary material, Figure S12B).

Next, we expanded our studies of PDGFRα inhibition to *mdx* mice in order to evaluate whether crenolanib can reduce the pro-fibrotic response of collagen-producing cells *in vivo*. Crenolanib or the vehicle was administered for 4 weeks in the drinking water *ad libitum*, beginning at 8 weeks of age, a timeframe when elevated numbers of *PDGFR*α-expressing cells are evident (supplementary material, Figure S6), and just prior to the development of fibrosis in *mdx* diaphragms [15,40]. Based on published pharmacokinetic data [58], the concentration of crenolanib in the drinking water was set at 0.03 mg/ml, so that animals received an approximate daily dose of 5 mg/kg that would achieve inhibition of PDGFRα. Control mice were treated with an equal amount of the vehicle (DMSO) in their drinking water. Following 4 weeks of treatment, muscle strength was initially tested by *ex vivo* myography (Figure 6A), an assay commonly used to measure functional improvement in *mdx* muscles [59]. Crenolanib-treated *mdx* mice generated significantly greater isometric force in both EDL muscles and diaphragm strips.

To understand the factors and mechanisms that contributed to this functional gain, we examined changes in the muscles' connective tissue. Histopathology of diaphragm muscles showed reduced collagen deposition, assessed in serial cross-sections as the percentage of Picrosirius red staining (Figure 6B). Whole tissue analysis indicated a significant reduction in the relative level of the *Col3a1* transcript (Figure 6C). In contrast, the relative expression of *Pdgf* receptors and ligands did not change (Figure 6C), suggesting that the treatment does not result in the transcriptional ablation of the pathway. Accordingly, histological quantification of GFP and type III collagen immunofluorescence confirmed a reduction of collagen type III in crenolanib-treated diaphragms, whereas the number of Col1a1-GFP+ cells did not significantly change in comparison to vehicle-treated diaphragms (supplementary material, Figure S13). Western blot analysis of diaphragm muscles confirmed a significant decline in collagen type III with crenolanib. Fibronectin, which also accumulates with fibrosis and can be present in necrotic fibres

Figure 4. Col1a1-GFP+ cells and *Pdgfra* expression increase with muscle injury to reconstitute the damaged connective tissue. (A) Images (left panels) of uninjured and cardiotoxin (CTX) injured TA muscles from a wt:*Col1a1-GFP:Myf5*$^{nlacZ/+}$ dual reporter mouse (1.5-month-old male) showing the response of GFP+ collagen-producing cells alongside X-gal + myogenic precursors to muscle injury. In uninjured muscle, Myf5^{nlaZ}-expressing cells (arrowhead) are separate from Col1a1-GFP+ cells. In contrast, X-gal + and GFP+ cells are abundant and in close proximity in damaged regions of muscle at the peak of myogenic cell response, 3 days following CTX injection. RT-qPCR analysis (right panel) indicates the elevation of pro-collagen mRNA levels in response to CTX injury and the decline as regeneration concludes 14 days following injury. (B) Images (left panels) of PDGFRa-nGFP+ cells in the endomysium and perimysium near vessels (arrowhead) and collagen type I in normal muscle and response 3 days following cardiotoxin (CTX) injury (shown is TA muscle from a 1.5-month-old wt:*PDGFRa-nGFP* male mouse). RT-qPCR analysis (right panel) for *Pdgfra* and *Pdgfa* ligand in response to CTX injury and repair. Note levels are reduced close to uninjured levels as regeneration concludes, 14 days post injury. $N = 3$ TA muscles from 12-month-old males per time point. $^{\#}p = 0.08$; $^{*}p \leq 0.05$; $^{**}p < 0.005$ by single factor ANOVA. Bars show mean ± SEM. (C, D) Representative photomicrographs and quantification of Picrosirius red and Fast Green-stained quadriceps muscles from 3-month-old Cre-flox mice. Animals received daily intraperitoneal injections of tamoxifen for five consecutive days. Fourteen days following the last dose of tamoxifen, muscles were injected with cardiotoxin (CTX) and collected 21 days post-injury. Contralateral muscles were used as the uninjured controls. Note that tamoxifen-induced, Cre-mediated expression of the mutant (*D842V*), constitutively active form of PDGFRα at the endogenous locus (left column) resulted in fibrosis by day 21 following CTX injury, whereas expression of the constitutively active mutant (*D536A*) form of PDGFRβ resulted in much milder fibrosis (middle left) and incomplete regeneration following injury. In the absence of Cre recombination, neither PDGFR flox resulted in fibrosis (middle right and right). Scale bars = 50 μm.

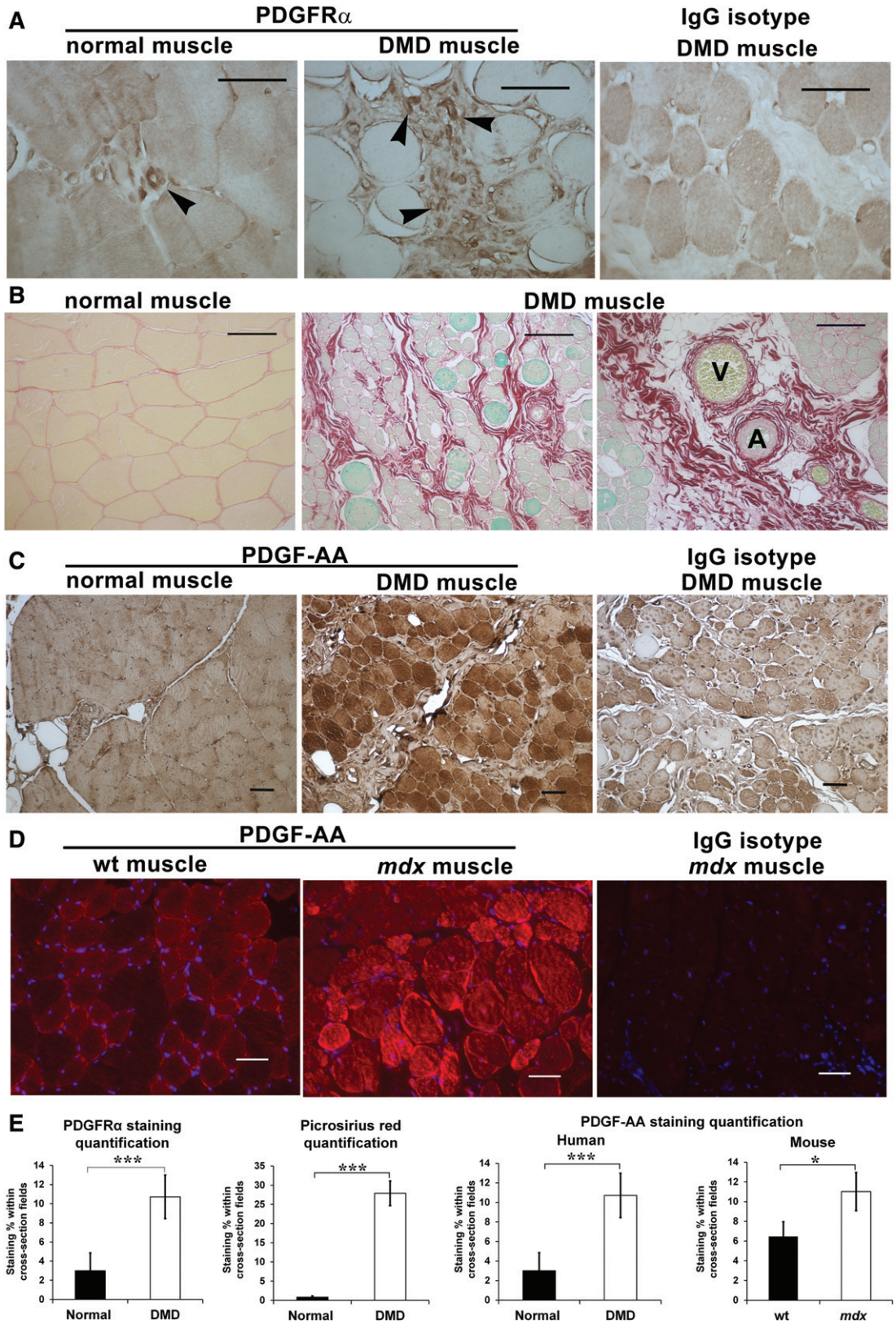

Figure 5. PDGFRα + cells and the PDGF-AA ligand are up-regulated in human DMD muscles. (A) PDGFRα + immunohistochemistry (brown) reveals increased numbers of PDGFRα + cells (arrowheads) in biopsies of DMD muscles compared with healthy quadriceps from males aged 8–10 years ($N = 3$). (B) Picrosirius red and Fast Green staining of the same muscles shows more collagen in the endomysium and perimysium, particularly near vessels, venules (V) and arterioles (A), where PDGFRα + cells are observed in human and mouse muscles. (C) PDGF-AA immunohistochemistry (brown) on the same DMD and normal muscle tissue sections reveals a strong presence of this ligand in dystrophic muscles, expressed primarily in muscle fibres. (D) PDGF-AA detection by immunofluorescence was also more intense in *mdx* than in wild-type male mouse quadriceps at 18 and 24 months, respectively. Scale bars = 50 µm. (E) Quantification of staining indicates significant increases in PDGFRα and fibrosis in DMD muscle in addition to elevated PDGF-AA staining in *mdx* and DMD muscles. $^{*}p < 0.05$; $^{***}p < 0.0005$. Bars indicate mean ± SEM.

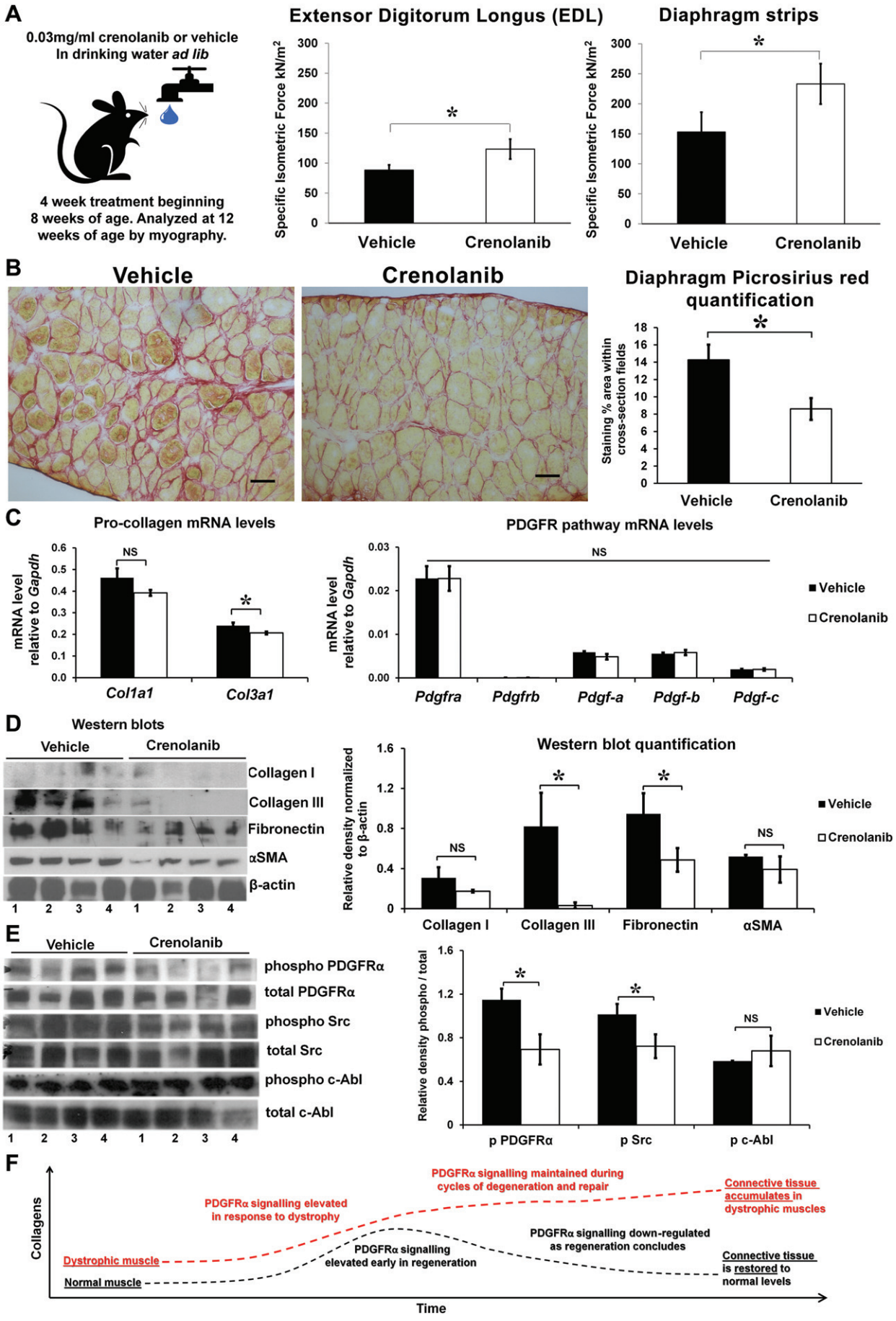

Figure 6. Legend on next page.

(Figure 1C), was also reduced (Figure 6D). An attenuation of necrotic areas was also noted with crenolanib (supplementary material, Figure S14). Such reductions in connective tissue and fibrotic components correlated with reduced phosphorylation of PDGFRα and its intracellular downstream effector, Src (Figure 6E). Indeed, phosphorylated Src levels are increased in *mdx* diaphragms in comparison to same-age wild-type diaphragms (supplementary material, Figure S15). In contrast, phosphorylation of c-Abl, which is the main target of imatinib [15], did not change with crenolanib. Of note, a reduction in cardiac fibrosis and PDGFRα phosphorylation was also detected in the hearts of crenolanib-treated *mdx* mice (supplementary material, Figure S16).

To discern whether a reduction in fibrosis permitted increased regeneration, we evaluated myogenic factors in crenolanib-treated and vehicle-treated *mdx*:*Col1a1-GFP* muscles. As previously shown, collagen-producing cells are involved in regeneration (Figure 4A, B). In these experiments, we again observed Col1a1-GFP+ cells in close proximity to regenerating fibres expressing sarcoplasmic αSMA, which were greater in number and size with crenolanib (supplementary material, Figure S17). The Col1a1-GFP+ cells were more abundant in regenerating areas of crenolanib-treated diaphragm and EDL muscles (supplementary material, Figure S18A). Whole tissue levels of *Acta2* transcripts in crenolanib-treated diaphragm muscles were increased, in keeping with the reduction in fibrosis being more permissive for regeneration (supplementary material, Figure S18B). Crenolanib-treated muscles showed significantly increased expression of the myogenic regulatory factor *Pax7*, a specific marker of satellite cells, but not of *Myf5* or *Myod*, which are expressed by satellite cells and myoblasts [51,60,61] (supplementary material, Figure S18B). Such an increase in *Pax7* transcripts suggests that the amelioration of fibrosis may result in greater satellite cell renewal during the course of treatment [60].

Discussion

Fibrosis in muscular dystrophy is widely recognized as a barrier to regeneration, yet remains understudied. Utilizing robust genetic models and molecular methods, in our present study we have identified an important role for PDGFRα signalling in collagen-producing cells during damage to connective tissue. In the absence of disease, collagen-producing cells restore the connective tissue in damaged muscles. This process is associated with transient elevations of mRNA levels for pro-collagens and PDGFRα signalling components (the receptor and ligand), as Col1a1-GFP+ cells respond to injury in a coordinated manner with myogenic precursors. In contrast, dystrophic muscles undergo continuous cycles of degeneration/regeneration, which maintains the response of Col1a1-GFP+ cells and PDGFRα signalling. Increased PDGFRα phosphorylation [15] and PDGFRα expression by mesenchymal progenitors that have potential to express collagens [4,5,7,8] suggest a role for this pathway in the pathology of muscular dystrophy.

To date, several populations of muscle cells, including FAPs and myogenic, haematopoietic, and endothelial cells, have been reported to express collagens and promote fibrosis [6,13]. Our results indicate that the majority of Col1a1-GFP+ cells share a profile similar to FAPs (Sca1+, PDGFRα+, CD45−), suggesting that these populations are not mutually exclusive. In addition, a very small number (<3%) of Col1a1-GFP+ cells expressed endothelial (CD31+, CD45−) or haematopoietic (CD45+) markers. Irrespective of cellular origin or classification, the pro-fibrotic transition of muscle cells is frequently associated with PDGFRα expression. This tendency was also observed in our study, where the majority of Col1a1-GFP+ cells were PDGFRα + in dystrophic but not normal muscles. However, whether PDGFRα-expressing cells expanded or originated from various cell types that up-regulate PDGFRα with disease or injury remains to be elucidated. Careful fate-mapping of PDGFRα expression in conjunction with a reporter of collagen expression will be required to further delineate the origin and heterogeneity of pro-fibrotic cells.

The expression of PDGFRα by collagen-producing cells *in vivo* and the presence of the PDGF-AA ligand in dystrophic muscles indicate a paracrine role for PDGF-AA, which remains activated in response to chronic injury. PDGF-AA is expressed by developing muscles but is absent in the somites of *Myf5*-null mutants [62]. Consequently, it is entirely possible that PDGF signalling in muscular dystrophy is a reactivation of the development programme that occurs in response to muscle repair. Therefore, we hypothesize

Figure 6. Inhibition of PDGFRα signalling and its downstream effector Src, but not c-Abl, results in decreased fibrosis and improved muscle strength of *mdx* muscles. (A) Schema (left panel) depicting treatment of *mdx* male mice with crenolanib or vehicle ($N = 6$ per group). Myography (centre and right) of these treated mouse muscles records improved muscle strength in extensor digitorum longus (EDL) and diaphragm muscles following crenolanib. (B) Images and quantification of collagen deposits (fibrosis) detected by Picrosirius red staining following vehicle or crenolanib ($N = 4$ per group) treatment in *mdx* diaphragms. Scale bars = 50 μm. (C) RT-qPCR for *Col1a1* and *Col3a1* mRNA showed a modest reduction in relative abundance. RT-qPCR for mRNAs of PDGFs and PDGFRs showed no changes in relative abundance following crenolanib ($N = 4$ per group). (D) Western blots and normalized densitometry indicating a significant reduction in type III collagen and fibronectin in diaphragms from crenolanib-treated *mdx* mice ($N = 4$ per group). (E) Western blots of treated diaphragms show that crenolanib reduced p-PDGFRα and p-Src, but not p-Abl. $^*p \leq 0.05$ by Student's *t*-test. Bars indicate mean ± SEM. (F) Summary and hypothesis: in contrast to acute PDGFRα activation in non-diseased muscles that repair and lack fibrosis, chronic activation of PDGFRα signalling in muscular dystrophy potentiates connective tissue accumulation. To represent the pathogenesis in muscular dystrophy, the *y*-axis depicts collagen accumulation versus time along the *x*-axis.

that continuous activation of PDGFRα signalling in collagen-producing cells and the resultant fibrosis are a consequence of the chronic injury and repair that occur in dystrophic muscles. In contrast, non-diseased muscles down-regulate PDGFRα signalling once the muscles' connective tissue has been restored, during the process of regeneration (modelled in Figure 6G). In addition to the aforementioned findings, the capability of PDGFRα signalling to promote fibrosis when constitutively activated during the repair process in non-diseased muscles (Figure 6G) supports our hypothesis that excessive PDGFRα signalling promotes fibrosis and prevents normal repair. Consequently, inhibition of PDGFRα with crenolanib resulted in reduced fibrosis and functional improvement of *mdx* muscles (Figure 6A–F). Such results not only implicate PDGFRα signalling in the fibrotic response of collagen-producing cells but also present a novel target for reducing connective tissue accumulation in patients with DMD.

Lemos *et al* [6] recently demonstrated another paracrine role mediated by macrophages, which in normal regenerating muscle secreted TNF to induce apoptosis of FAPs. In contrast, they showed that in dystrophic muscle, macrophages secreted TGF-β, which prevented apoptosis but induced the fibrogenic programme of FAPs. Whether macrophages or muscle cells in addition to muscle fibres regulate PDGFRα signalling in FAPs and/or Col1a1-GFP+ cells remains an important question worth pursuing.

Importantly, the intracellular signalling cascade by which PDGFRα stimulation leads to collagen production remains unknown. The reported reduction of *mdx* muscle fibrosis by imatinib has been attributed to PDGFR and c-Abl tyrosine-kinase inhibition [15]. Our results indicate that selective inhibition of PDGFRα, independent of c-Abl, can reduce fibrosis in *mdx* muscles and improve function (Figure 6). Herein, we demonstrated that Src, a direct downstream target of PDGFRα signalling, is increased in *mdx* diaphragms and that crenolanib treatment reduces phosphorylated Src. Recently, Pal *et al* [63] showed that oxidative stress results in persistent activation of Src in *mdx* muscles and impairs autophagy via mTOR. Crenolanib has 100-fold higher selectivity for PDGFR than other tyrosine kinases including Src and c-Abl [55]. Thus, the observed reduction of phosphorylated Src following crenolanib treatment is mediated by direct inhibition of PDGFRα. Further studies are needed to define the signalling cascade by which PDGFRα-Src mediates the pro-fibrotic response of collagen-producing cells in skeletal muscles. Although c-Abl may also potentiate collagen transcription, c-Abl inhibition with imatinib, and to a lesser extent with nilotanib, has been associated with cardiomyocyte toxicity and heart failure in cancer patients [57,64]. Such a side effect may compound the cardiomyopathy prevalent in the majority of DMD patients [65]. Therefore, anti-fibrotic therapies that selectively target PDGFRα-Src signalling may prove efficacious and safer than broad-spectrum tyrosine-kinase inhibitors.

To conclude, we have further defined the role of PDGFRα +, Sca1+, CD45− mesenchymal progenitors in skeletal muscle, which become activated after injury to become pathological matrix-forming cells. In settings of excessive PDGFRα signalling, these cells are a major population of matrix-forming fibroblasts which promote muscle fibrosis and inhibit normal regeneration. Crenolanib, a specific PDGFRα/β inhibitor, reduces fibrosis in a mouse model of DMD and in turn improves muscle function.

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Author contribution statement

The authors contributed in the following way: writing and editing: NI, JSD, and MR; study design: NI, MR, and JSD; experimentation: NI, AH, AM, and KJ; data analysis: NI, MR, JSD, AH, AP, and KJ.

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Supplementary figure legends

Figure S1. Col1a1-GFP+ cells are expanded in areas of perimysial fibrosis of *mdx* quadriceps muscle, where type III and type VI collagens are deposited

Figure S2. GFP+ cells also expand in areas of type III and type VI collagen deposition in the myocardium of *mdx:Col1a1-GFP* mice

Figure S3. Sca1 is concentrated in the PDGFRα + portion of the *Col1a1-GFP*+ population in both wild-type and *mdx* diaphragms

Figure S4. Col1a1-GFP+ cells in the heart and diaphragm share a similar molecular profile

Figure S5. PDGFRα-nGFP+ cells are concentrated in degenerating and regenerating regions of *mdx* muscles

Figure S6. Fate-mapping reveals that PDGFRα-expressing cells are prominent at the onset of dystrophy in *mdx* diaphragm muscle

Figure S7. Col1a1-GFP+ cells are adjacent to both myogenic cells and regenerating fibres following acute cardiotoxin injury in healthy muscle

Figure S8. Histological analysis of mutant muscles shows reduced fibre size with excessive PDGF signalling following CTX injury

Figure S9. Tamoxifen-treated *PDGFRa-CreER;mT/mG loxP* mice show activation of the GFP reporter in both uninjured and injured muscle

Figure S10. Vehicle-treated *PDGFRa-CreER;mT/mG loxP* mice show no evidence of recombination in uninjured or injured muscle

Figure S11. Single fibre isolations and co-culture with Col1a1-GFP+ cells indicate that *mdx* fibres promote fibrosis by their production of PDGF-AA

Figure S12. Crenolanib inhibits PDGF-AA-mediated expression of pro-collagen mRNAs and phosphorylation of the PDGFRα in primary cultured Col1a1-GFP+ cells

Figure S13. Crenolanib attenuates type III collagen accumulation in *mdx* diaphragms

Figure S14. Necrotic regions are smaller with crenolanib treatment

Figure S15. The phosphorylation of Src is increased in *mdx* diaphragms

Figure S16. Crenolanib reduces PDGFRα signalling and fibrosis in *mdx* hearts

Figure S17. The number and size of regenerating fibres are greater with crenolanib treatment

Figure S18. Crenolanib improves regeneration in *mdx* skeletal muscles

Table S1. Mouse strains used

Table S2. Antibodies and lectins used on tissue sections

Table S3. FACS antibodies and streptavidin conjugates

Table S4. RT-qPCR primer sequences

Table S5. Antibodies used for western blotting