



Fibrogenic Cell Plasticity Blunts Tissue Regeneration and Aggravates Muscular Dystrophy

Patrizia Pessina,¹ Yacine Kharraz,¹ Mercè Jardí,¹ So-ichiro Fukada,² Antonio L. Serrano,¹ Eusebio Perdiguero,¹ and Pura Muñoz-Cánoves^{1,3,*}

¹Cell Biology Group, Department of Experimental and Health Sciences (DCEXS), Pompeu Fabra University (UPF), CIBER on Neurodegenerative Diseases (CIBERNED), Dr. Aiguader, 88, 08003 Barcelona, Spain

²Laboratory of Molecular and Cellular Physiology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

³Institució Catalana de Recerca i Estudis Avançats (ICREA), Passeig Lluís Companys, 23, 08010 Barcelona, Spain

*Correspondence: pura.munoz@upf.edu

<http://dx.doi.org/10.1016/j.stemcr.2015.04.007>

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SUMMARY

Preservation of cell identity is necessary for homeostasis of most adult tissues. This process is challenged every time a tissue undergoes regeneration after stress or injury. In the lethal Duchenne muscular dystrophy (DMD), skeletal muscle regenerative capacity declines gradually as fibrosis increases. Using genetically engineered tracing mice, we demonstrate that, in dystrophic muscle, specialized cells of muscular, endothelial, and hematopoietic origins gain plasticity toward a fibrogenic fate via a TGF β -mediated pathway. This results in loss of cellular identity and normal function, with deleterious consequences for regeneration. Furthermore, this fibrogenic process involves acquisition of a mesenchymal progenitor multipotent status, illustrating a link between fibrogenesis and gain of progenitor cell functions. As this plasticity also was observed in DMD patients, we propose that mesenchymal transitions impair regeneration and worsen diseases with a fibrotic component.

INTRODUCTION

Successful regeneration after tissue injury requires timely coordinated actions of diverse cell types. In skeletal muscle, in response to acute damage, the muscle stem cell (satellite cell) progeny gives rise to new regenerating myofibers, aided by the concerted action of specialized cells, such as infiltrating bone-marrow-derived inflammatory cells, which phagocytose tissue debris and provide pro-myogenic growth factors and cytokines; fibrogenic stromal cells such as fibroblasts and adipogenic progenitors (FAPs), which provide transient matrix support; and angiogenic cells that vascularize the newly formed muscle tissue (Abou-Khalil et al., 2010; Mounier et al., 2011). In chronically damaged muscle, however, this coordination is lost, leading to deficient regeneration (Serrano et al., 2011). In the yet incurable Duchenne muscular dystrophy (DMD), caused by loss of the myofiber protein dystrophin, successive cycles of tissue degeneration and regeneration lead to an eventual muscle regenerative failure and replacement of dystrophic muscle by fibrotic tissue, resulting in respiratory failure and early death (Mann et al., 2011; Stedman et al., 1991; Wallace and McNally, 2009).

Cell plasticity (i.e., the capacity of cells to change their phenotypic properties) is inherent to organismal development and is becoming increasingly associated with tissue remodeling in the adult (Medici and Kalluri, 2012; Nieto, 2013). Mesenchymal transitions (particularly epithelial- and endothelial-to-mesenchymal transitions, EMTs and

EndMTs, respectively) are connected both to fibrotic pathologies and cancer progression of distinct etiologies, affecting organs such as liver, lung, heart, or kidney (Medici and Kalluri, 2012; Nieto, 2013; Nieto and Cano, 2012; Zeisberg and Kalluri, 2013). Lineage-tracing and fate-mapping strategies have precisely determined and quantified the source of fibrogenic cells in fibrotic kidney, underscoring the relevance of EMT, EndMT, and bone-marrow-derived cells to this organ's fibrosis (LeBleu et al., 2013). Incomplete EMT also can occur in tumors, with cells acquiring mesenchymal properties without undergoing the full EMT as it also occurs in embryos, where intermediate phenotypes have been described in different contexts (Nieto, 2011, 2013; Nieto and Cano, 2012). These incomplete transitions implicate a change in cellular functions and behavior. In skeletal muscle, studies on cell plasticity during repair are emerging. In addition to resident interstitial fibroblasts and FAPs, which are considered the major producers of the collagen-rich extracellular matrix (ECM) in injured muscle and in young dystrophic muscle (Joe et al., 2010; Mann et al., 2011; Uezumi et al., 2011, 2014), perivascular progenitor cells transiently produce collagen in response to acute muscle damage, but disappear as regeneration advances (Dulauroy et al., 2012). Similarly, depletion of macrophages or age-induced Wnt signaling in acutely injured muscle can divert vascular and myogenic cell fates, respectively (Brack et al., 2007; Zordan et al., 2014). However, whether cell plasticity occurs in dystrophic muscle and how it affects disease progression have remained elusive.



Recently, fibrogenesis from muscle cells has been reported in DMD (Biressi et al., 2014).

Here we demonstrate that specialized cells of muscular, endothelial, and hematopoietic origins acquire mesenchymal-fibrogenic traits in dystrophic muscle, with this cellular plasticity being particularly associated with advanced DMD stages. The mesenchymal-fibrogenic plasticity of these cells is induced by increasing TGF β signaling in dystrophic muscle with aging, and results in the loss of cell identity, thus precluding normal regenerative functions. Together, our findings suggest that, during efficient tissue repair, specialized cells preserve their lineage identity by avoiding entrance into a mesenchymal-like/fibrogenic state. This protection is lost in chronic degenerative conditions such as DMD.

RESULTS

The levels of TGF β and downstream signaling mediators (activated SMAD2/3) increase in muscle of dystrophic mdx mice with age, correlating to reduced regeneration, angiogenesis and function, and higher fibrosis extent (Ardite et al., 2012; Kharraz et al., 2014; Mann et al., 2011; Vidal et al., 2008; Figure 1A; Figures S1A and S1B). Inflammatory cells and FAPs appeared as the principal sources of TGF β in dystrophic muscle (Figure S1C). Higher levels of this pathway also were found in muscle of wild-type (WT) mice after laceration (a severe injury model that induces persistent degeneration and more sustained fibrosis) than after cardiotoxin (CTX) injury (in which collagen-rich ECM is transient and full regeneration and muscle function are achieved rapidly) (Figures S1D and S1E). In agreement with the profibrotic role of TGF β , exogenous delivery of TGF β to CTX-injured WT muscle or dystrophic muscle of young mdx mice delayed regeneration and vascularization, while promoting fibrogenesis. This suggests that TGF β inhibits myogenic and angiogenic capacity of muscle stem cell (satellite cell)-derived myoblasts and endothelial cells, respectively, while promoting matrix accumulation (Figures S1F and S1G). Consistent with this, freshly isolated WT satellite cells were unable to fuse into myotubes in differentiation medium (DM) in the presence of TGF β (Figure S2A), correlating with gain of expression of fibrogenic genes (*α Sma*, *Collagen I*, *Eda-Fibronectin*, or *Timp1*) and loss of myogenic gene expression (*Myf5* and *Pax7*) after a 10-day treatment (Figure S2B). Likewise, endothelial cells isolated from skeletal muscle could not form angiotubes in vitro after a 10-day TGF β treatment period (Figure S2C), consistent with loss of expression of endothelial genes (*Cd31* and *Tie1*) and de novo acquisition of fibrogenic traits (Figure S2D). These results indicate that TGF β induces the loss of identity of muscle-resident

myogenic and endothelial cells by promoting their switch into matrix-producing fibrogenic cells, thus precluding their bona fide functions.

To further understand this cellular plasticity process induced by TGF β , we performed a microarray gene expression analysis of satellite cells treated (or not) with TGF β for 4 days (before achieving maximal levels of fibrogenic conversion, i.e., expression of *α Sma* [Figure S2B]). Gene ontology functional annotation of genes upregulated in TGF β -treated myogenic cells, compared to non-treated cells, showed enrichment in mesenchymal-fibrogenic functions (Figure S3A). Moreover, after comparison with a curated list of mesenchymal progenitor cell-specific transcripts (Kubo et al., 2009), we identified a group of mesenchymal cell-specific genes induced by TGF β (Figure S3A). qRT-PCR analysis of a TGF β cell-treatment kinetics experiment validated the expression of mesenchymal progenitor genes at intermediate time points (Figure 1B). These mesenchymal genes were also significantly upregulated in endothelial cells isolated from skeletal muscle in response to identical TGF β treatment, prior to larger acquisition of fibrogenic traits (Figure 1B; see scheme in Figure 1C).

To prove whether this gain of mesenchymal gene expression translated into de novo functional cellular multipotency (i.e., potential to differentiate into distinct cellular fates), satellite cells and endothelial cells were treated with TGF β for 3 days and further incubated with osteogenic or adipogenic medium for 7–14 days, or they continued to be treated with TGF β for 7 extra days. Notably, cells pretreated with TGF β showed induced expression of adipocyte, osteoblast, or fibrogenic traits under their respective differentiation regimes (Figures 1D and 1E; Figure S3B); in contrast, cells that had not been pretreated with TGF β did not undergo any of these conversions under identical differentiation conditions (Figures 1D and 1E). These results suggest that these two specialized cell types (myogenic and endothelial cells) gain expression of mesenchymal genes during the plastic process toward a more mature fibrogenic fate in response to TGF β ; furthermore, these cells exhibit multipotency under adequate culture conditions (see scheme in Figure 1C). Of note, TGF β was capable of inducing the expression of transcription factors and microRNAs associated with mesenchymal transitions in myogenic and endothelial cells (Figures S3C and S3D). In particular, *Mir21* induction appeared to mediate TGF β -induced fibrogenesis in both cell types (Figure 2), reinforcing this *Mir* as a fibrogenic effector of TGF β action (Acuña et al., 2014; Ardite et al., 2012; Kumarswamy et al., 2012).

Since TGF β levels and signaling are elevated in aged dystrophic muscle (Figure 1A), we next investigated whether endothelial and satellite cells also undergo plastic fibrogenesis in vivo. To this end, we generated endothelial

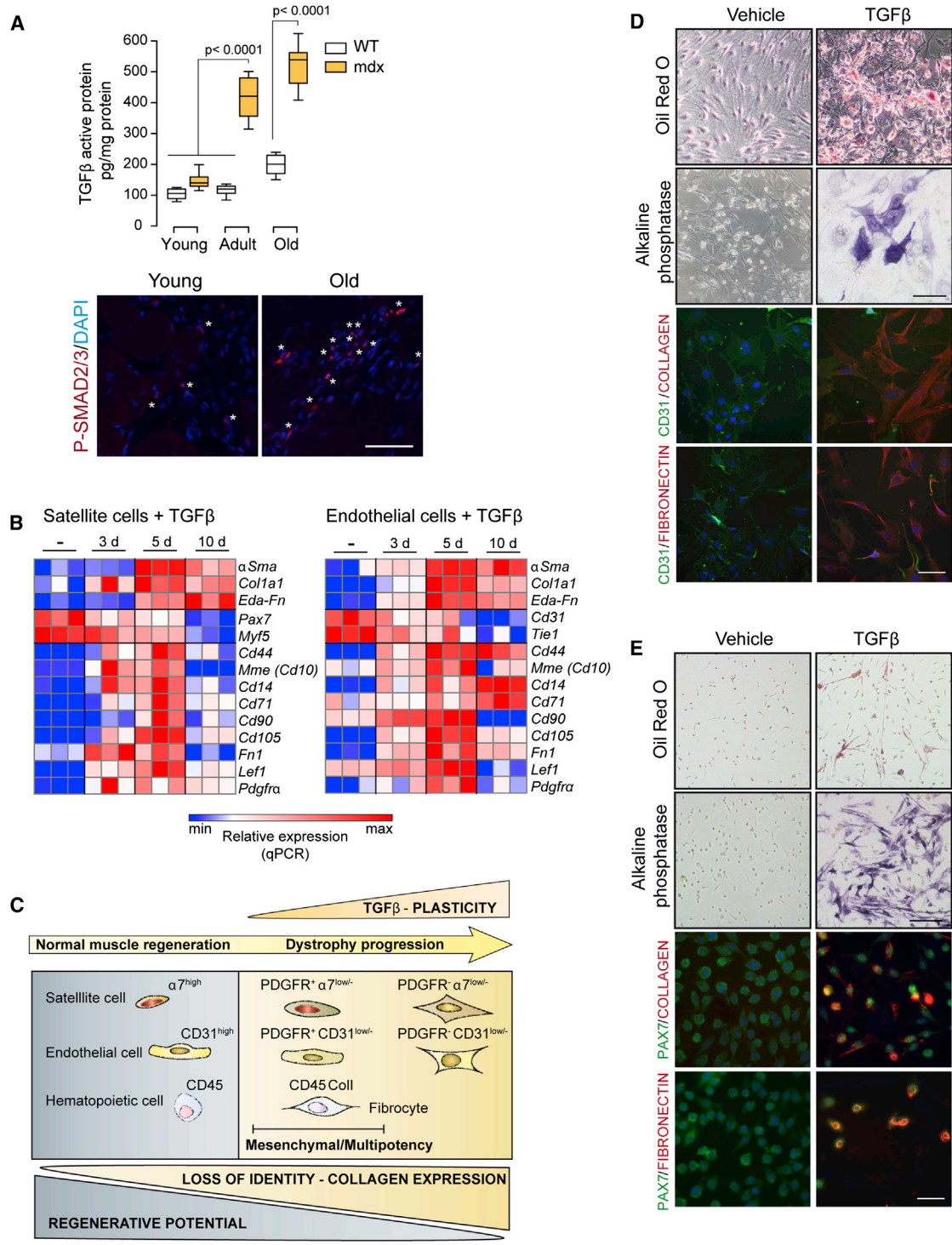


Figure 1. Increased TGFβ Signaling at Advanced Muscular Dystrophy Stages and TGFβ-Induced Cellular Plasticity

(A) Active TGFβ protein levels (ELISA) in limb muscles of mdx mice (C57BL/6 background) at distinct ages: young (2–3 months), adult (6–10 months), and old (18–24 months). Data correspond to the mean ± SEM values; n = 10 for each group. Non-parametric Mann-Whitney U test was used for comparison. Representative pictures of phosphorylated SMAD2/3 (P-SMAD2/3) protein immunostaining in mdx muscles are shown. *P-Smad2/3-positive cells. Scale bar, 50 μm.

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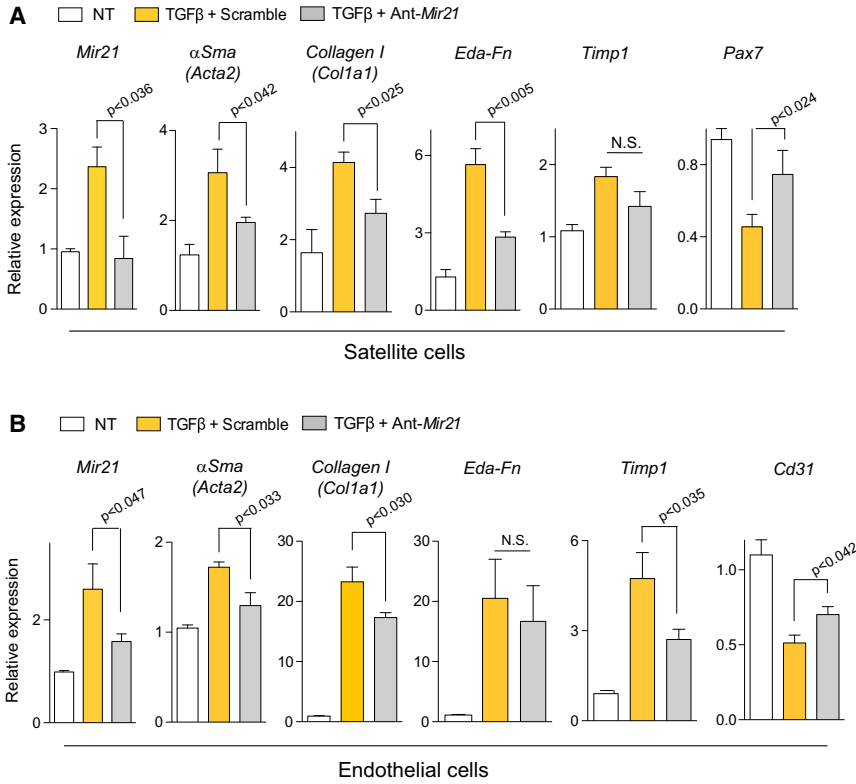


Figure 2. *Mir-21* Mediates TGFβ-Induced Fibrogenesis of Satellite Cells and Endothelial Cells

(A and B) Satellite cells (A) and endothelial cells (B), transfected with Ant-*Mir-21* or Scramble oligomiR, were treated with TGFβ for 8 days, and subjected to qRT-PCR for fibrogenic, myogenic or endothelial markers, respectively. Values are mean ± SEM; n = 3 independent experiments. Unpaired t test was used for comparison.

and satellite cell-lineage-tracing mice in two mdx dystrophic backgrounds, DBA/2 and C57BL/6, which develop substantial fibrosis in limb muscles at adult (6–10 months) or old (after 18 months) age, respectively (Fukada et al., 2010; Figures S4A and S4B). First, *Ve-Cad-Cre^{ER}/YFP* mice (obtained by intercrossing of *Cdh5(Ve-Cad)-Cre^{ER}* and *Rosa26R-YFP* mice, in which the expression of Cre is induced exclusively in adult endothelial cells expressing *Ve-Cadherin* upon tamoxifen administration) were intercrossed with mdx/DBA/2 mice, generating *Ve-Cad-Cre^{ER}/YFP/mdx* triple-mutant mice, which were aged until

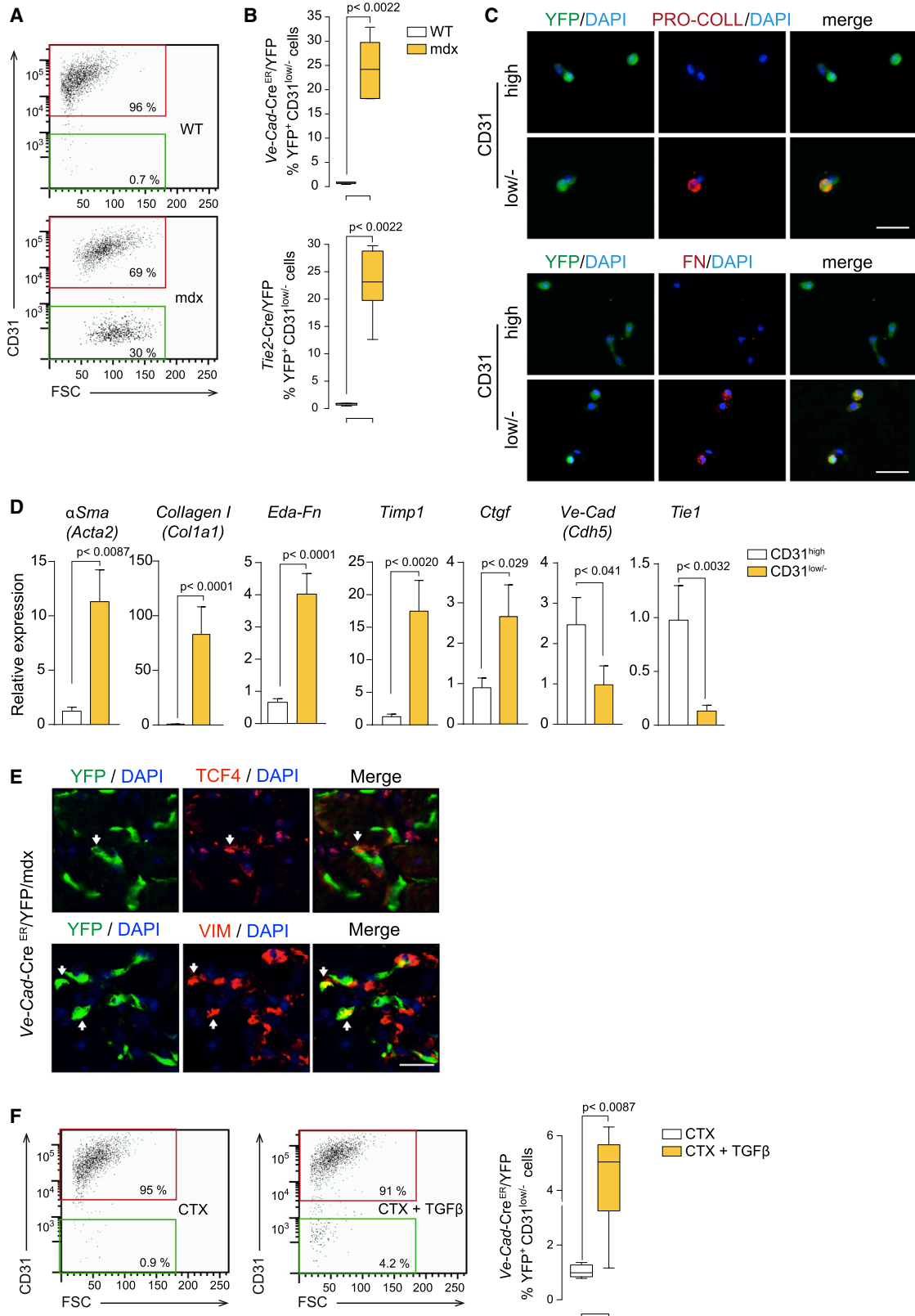
advanced fibrogenic stages (i.e., 6–10 months). A second endothelial genetic tracing line, *Tie2-Cre/YFP* mice (obtained by intercrossing of *Tie2-Cre* and *Rosa26R-YFP* mice, in which the *Tie2* gene is expressed by endothelial cells from early vascular development), was bred into the mdx/C57BL/6 dystrophic background, generating *Tie2-Cre/YFP/mdx* triple-mutant mice, which were subsequently aged up to 18–24 months for fibrosis development.

Use of the CD31 endothelial cell marker in a YFP⁺-based sorting protocol permitted discrimination between bona fide endothelial cells (YFP⁺CD31^{high}) and cells of

(B) Heat map representing qRT-PCR of fibroblastic, myogenic, and mesenchymal stem cell marker expression in satellite cells or endothelial cells treated with TGFβ for the indicated time points (days). Values are mean ± SEM; n = 3 independent experiments.

(C) Summary scheme representing the process of fibrogenic plasticity of myogenic, endothelial, and hematopoietic cells in dystrophic and injured skeletal muscle. Scheme is inferred from results using satellite cells and endothelial cells obtained from skeletal muscle, with or without treatment with TGFβ. Kinetics of fibrogenic cell plasticity induced by TGFβ is represented, with cells transiting via a mesenchymal progenitor cell-like state, which in turn is endowed with multipotent capacity. Similar plastic changes occur in muscle of lineage-tracing mice for endothelial and satellite cells in mdx dystrophic background (or in bone-marrow-transplanted mdx mice for hematopoietic cell tracing) and in WT muscle after CTX injury/TGFβ administration. Endothelial and satellite cells gain fibrogenic plasticity and lose their own identities and functions in vivo (become CD31^{low/-} or α7-integrin^{low/-}, and have reduced angiogenic and myogenic potential [not shown]). During this process, these cells express the mesenchymal progenitor marker PDGFRα and exhibit multipotency under adequate lineage-differentiation conditions (see D and E). We also detected fibrogenic cells of hematopoietic origin in dystrophic muscle (i.e., fibrocytes characterized by co-expression of CD45 and COLLAGEN I).

(D and E) Multipotency analysis of TGFβ pre-treated endothelial cells (D) and satellite cells (E), further cultured in adipogenic, osteogenic, and fibrogenic DM. Oil Red O (adipogenic), alkaline phosphatase (osteogenic), and COLLAGEN or FIBRONECTIN (fibrogenic) staining detect multi-lineage potential compared to non-pre-treated cells. Scale bars, 50 μm.



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endothelial origin that had reduced (or lost) expression of CD31 (YFP⁺CD31^{low/-}). The YFP⁺CD31^{high} cells represented the most prominent YFP⁺ cell population in *Ve-Cad-Cre^{ER}/YFP* and *Tie2-Cre/YFP* (WT) muscle (Figures 3A and 3B). In fibrotic-dystrophic muscle, however, a YFP⁺CD31^{low/-} cell population was induced (Figures 3A and 3B). Fibrogenic markers (*Collagen I* and *Fibronectin*) were found exclusively in the YFP⁺CD31^{low/-} cell fraction (Figure 3C), which accounted for approximately 20%–25% of the endothelial cell population (Figures 3A and 3B), and this was confirmed by qRT-PCR (Figure 3D), supporting the occurrence of cell plasticity characterized by the reduction of endothelial identity traits and acquisition of the fibrogenic gene program, at advanced muscular dystrophy. This conclusion was confirmed by the detection of cells double positive for fibrogenic and endothelial cell markers in muscle at late dystrophic stages (Figure S4C; Figure 3E), which coincided with the reduced vascularization and regeneration and increased fibrosis (Figure S1B). Furthermore, delivery of TGFβ to CTX-injured muscle of *Ve-Cad-Cre^{ER}/YFP* mice induced the presence of YFP⁺CD31^{low/-} fibrogenic cells (Figure 3F), although to a lesser extent than in dystrophic muscle (Figures 3A and 3B). qRT-PCR and immunofluorescence analysis confirmed the reduction of endothelial cell markers and gain of fibrogenic ones in the YFP⁺/CD31^{low/-}-sorted cells in TGFβ/CTX-injured WT muscles (Figures S4D and S4E).

Next, using a similar lineage-tracing strategy, we generated *Pax7-Cre/YFP* double-transgenic mice (obtained by intercrossing *Pax7-Cre* and *Rosa26R-YFP* mice, in which the expression of Cre is induced in *Pax7*-expressing muscle precursor cells) that were intercrossed with *mdx/C57BL/6* dystrophic mice, and the resulting *Pax7-Cre/YFP/mdx* triple-mutant mice were aged up to 18–24 months. We also used *Pax7-Cre^{ER}/YFP* mice (in which Cre is induced only in adult satellite cells upon tamoxifen administration) that were intercrossed with *mdx/DBA/2* mice, generating *Pax7-Cre^{ER}/YFP/mdx* triple-mutant mice, which were

aged until advanced fibrogenic stages at adult age (6–10 months). Double-labeling cell sorting for YFP and the satellite cell marker α7-INTEGRIN allowed us to discriminate between bona fide satellite cells (YFP⁺α7^{high}) and cells that had reduced or lost (YFP⁺α7^{low/-}) α7-INTEGRIN expression after CTX/TGFβ injury and in aged dystrophic mice. The YFP⁺α7^{low/-} cell population was induced specifically in CTX/TGFβ-injured muscle (compared to CTX-injured, or non-injured, muscle) of *Pax7-Cre^{ER}/YFP* mice (Figure 4A). This population was further increased (up to 11%–15%) in *Pax7-Cre^{ER}/YFP/mdx* muscle of adult age (BDA/2 background) or old age (C57BL/6 background) (Figure 4B; Figure S4F). Supporting the decline in myoblast cell identity in vivo, the expression of *Pax7* decreased, while fibroblastic gene transcripts increased in YFP⁺α7^{low/-} cells from *mdx* and TGFβ/injured WT muscles (Figures 4C and 4D). Furthermore, in myofiber explants, the number of bona fide satellite cells associated with each myofiber decreased in fibrotic *Pax7-Cre/YFP/mdx* mice, as indicated by the lower number of YFP⁺ cells expressing PAX7 or MYOGENIN (markers of quiescent/proliferating or differentiated myogenic cells, respectively), compared to age-matched WT myofibers (Figure 4E). Conversely, YFP⁺ cells in *mdx* myofiber explants gained expression of fibrogenic markers (Figure 4E). Co-expression of fibrogenic and myogenic proteins (or YFP) (Figure S4G; Figure 4F) further supported satellite cell fibrogenic plasticity in aged dystrophic muscle. Together, based on quantifications of these analyses (Figures 4B and 4E; Figure S4F), 11.7% of cells of myogenic origin gained fibrogenic traits in aged dystrophic muscle.

We subsequently tested the functional consequences of this fibrogenic plasticity in dystrophic muscle. The fluorescence-activated cell sorting (FACS)-isolated cells of endothelial and myogenic origins (YFP⁺) that had reduced (or lost) expression of CD31 or α7-INTEGRIN (YFP⁺CD31^{low/-} or YFP⁺α7^{low/-}) showed a severely impaired capacity to form angiotubes and myotubes in pro-angiogenic

Figure 3. Fibrogenic Plasticity of Endothelial Cells at Advanced Muscular Dystrophy Stages and in Response to TGFβ

(A) Representative FACS plots of CD31 expression in YFP⁺ cells from dystrophic *Ve-Cad-Cre^{ER}/YFP/mdx* (*mdx*) muscle compared to *Ve-Cad-Cre^{ER}/YFP* (WT) muscle. *Mdx* mice were in the DBA2 background and analyses were performed at adult age.

(B) Quantification of YFP⁺ cells with reduced/lost CD31 expression in *Ve-Cad-Cre^{ER}/YFP/mdx* mice in DBA2 background at adult age and in *Tie2-Cad-Cre/YFP/mdx* mice in C57BL/6 background at old age. Values are mean ± SEM; n = 6 animals for each group. Non-parametric Mann-Whitney U test was used for comparisons.

(C) Immunofluorescence analysis of FACS-isolated cells from (B): YFP⁺ cells that gain PRO-COLLAGEN and FIBRONECTIN expression while losing CD31 expression (CD31^{low/-}). Nuclei are stained with DAPI. Scale bars, 50 μm.

(D) qRT-PCR in FACS-isolated YFP⁺CD31^{low/-} cells from *Ve-Cad-Cre^{ER}/YFP/mdx* mice compared to YFP⁺CD31^{high} cells for the indicated fibroblastic and endothelial cell markers. Values are mean ± SEM; n = 6 independent experiments (mice) for each group. Unpaired t test was used for comparison.

(E) Representative immunostaining shows YFP⁺ cells co-expressing fibrogenic markers in *Ve-Cad-Cre^{ER}/YFP/mdx* mice. Scale bars, 25 μm.

(F) Representative FACS plots of CD31 expression in YFP⁺ cells isolated from CTX-injured and CTX/TGFβ-injured muscle (after 14 days of the initial injury [Figure S1F]) of *Ve-Cad-Cre^{ER}/YFP* mice, and quantification of YFP⁺ cells with reduced/lost CD31 expression. Values are mean ± SEM; n = 6 animals for each group. Non-parametric Mann-Whitney U test was used for comparison.

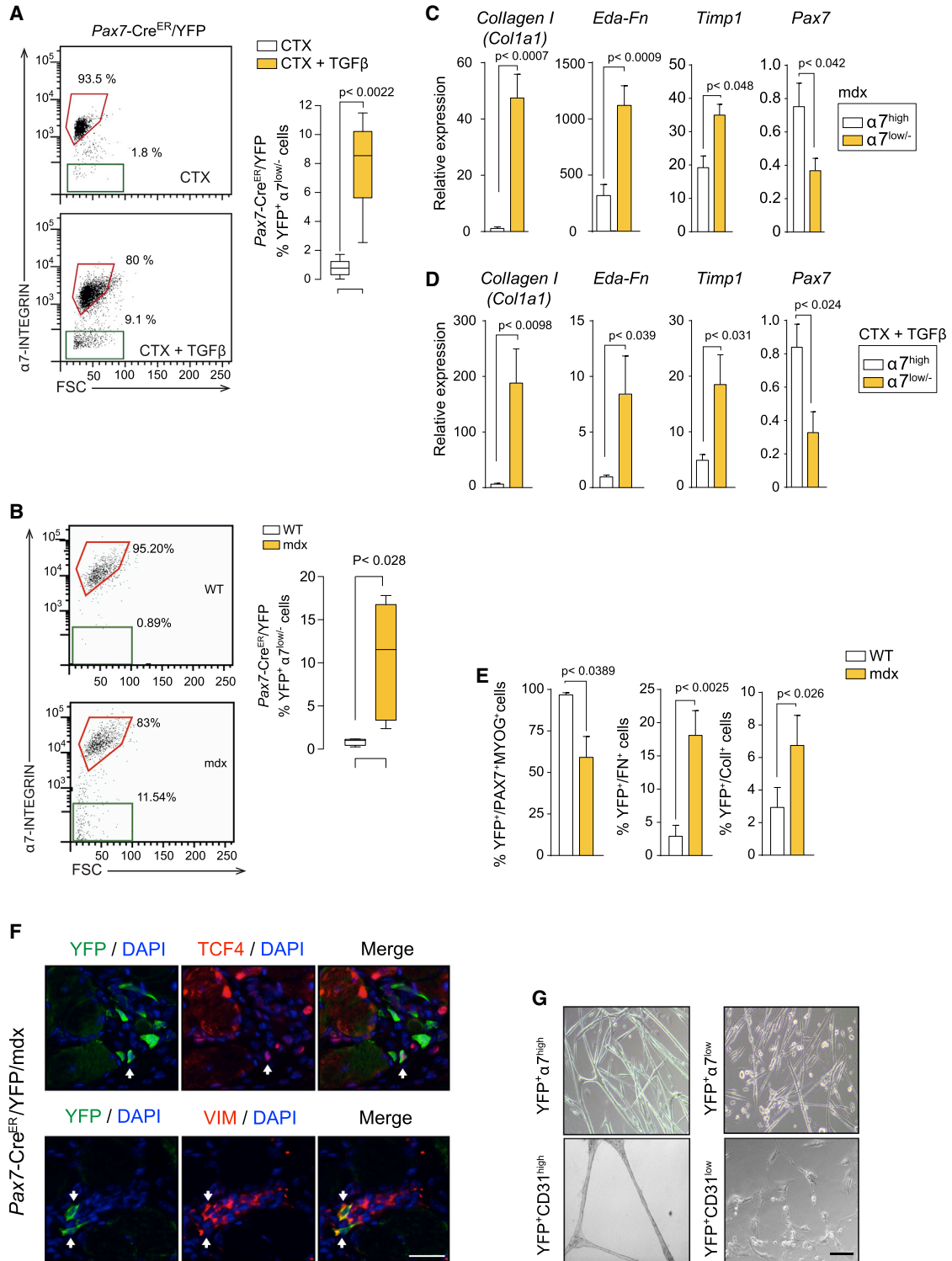


Figure 4. Fibrogenic Plasticity of Satellite Cells at Advanced Muscular Dystrophy Stages and in Response to TGF β

(A) Representative FACS plots of $\alpha 7$ -INTEGRIN expression in CTX-injured and CTX/TGF β -injured Pax7-Cre^{ER}/YFP muscles (as in Figure S1F) and quantification of YFP⁺ cells that have reduced/lost $\alpha 7$ -integrin expression. Values are mean \pm SEM; n = 6 animals for each group. Non-parametric Mann-Whitney U test was used for comparison.

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and pro-myogenic differentiation conditions, respectively (Figure 4G). Despite the significant fraction of both endothelial and myogenic cells undergoing fibrogenic plasticity in dystrophic muscle, surprisingly, each cell type only constituted about 2% of the bona fide active collagen-expressing cell population (i.e., active fibroblasts), based on intracellular collagen protein staining of the YFP⁺ CD31^{low/-} and YFP⁺α7^{low/-} cell populations, respectively (Figure S5A). Similarly, using a *Coll*-GFP reporter mice (in which GFP expression is under the control of the *Collagen I* promoter), only a low percentage of CD31⁺ and α7-INTEGRIN⁺ cells were found within the fibroblastic (GFP⁺) cell population (Figure S5B). These findings strongly suggest that, unlike other fibrotic organs, such as kidney (LeBleu et al., 2013), these fibrogenic changes do not lead to full and ample transformation into collagen-producing cells. Instead, this fibrogenic plasticity mainly precludes efficient myogenesis and angiogenesis and it impairs tissue repair.

We next investigated if endothelial and satellite cells also transit through intermediate mesenchymal progenitor states during the process of fibrogenesis in diseased muscle. To this end, we set up a FACS protocol based on the use of the cell surface mesenchymal progenitor marker PDGFRα (Chong et al., 2013; Pinho et al., 2013; Uezumi et al., 2010, 2014). A subpopulation of YFP⁺ cells that were low for CD31 or α7-INTEGRIN expression appeared positive for PDGFRα (YFP⁺PDGFRα⁺) in fibrotic muscles of dystrophic lineage-tracing mice (Figures 5A and 5B). The YFP⁺PDGFRα⁺CD31^{low/-} and YFP⁺PDGFRα⁺α7^{low/-} populations represented 14.6% and 16.3% of the fibrogenic YFP⁺CD31^{low/-} and YFP⁺α7^{low/-} cell fractions in adult mdx muscle, respectively (Figures 5A and 5B), and these percentages even increased in older mice (Figure S5C). As for the YFP⁺CD31^{low/-} and YFP⁺α7^{low/-} cell fractions, the PDGFRα⁺-expressing cell subpopulations were incapable of forming myotubes or angiotubes under appropriate differentiation conditions (not shown). The qRT-PCR analysis confirmed induction of mesenchymal progenitor markers in the freshly isolated YFP⁺PDGFRα⁺

CD31^{low/-} and YFP⁺PDGFRα⁺α7^{low/-} subpopulations from dystrophic muscle (Figure 5C). Thus, during the process of endothelial and satellite cell plasticity toward fibrogenesis, a fraction of cells shows mesenchymal progenitor traits. This was consistent with a subpopulation of YFP⁺ endothelial or myogenic cells also gaining PDGFRα⁺ expression in WT muscle subjected to CTX/TGFβ injury (Figure 5D). Consistent with the notion that TGFβ signaling is a driving cause for these plastic mesenchymal transitions, we could detect co-expression of activated SMAD2/3 in PDGFRα⁺/YFP⁺ cells in muscle of the distinct lineage-tracing mice (in various muscle degeneration/fibrosis paradigms) (Figure 5E; Figure S5D; data not shown) and in human DMD (see below).

To finally prove that these subpopulations of YFP⁺PDGFRα⁺ cells (from endothelial or myogenic origin) are indeed mesenchymal in nature, we tested their multipotency (the capacity to be coaxed to differentiate into distinct terminal fates: fat, bone, cartilage, or scar/fibrous) if exposed to adequate conditions. YFP⁺PDGFRα⁺ cells were FACS isolated from muscle of lineage-tracing dystrophic mice and subsequently cultured with osteogenic, adipogenic, or chondrogenic differentiation media or with TGFβ (for fibrogenic differentiation). In response to these treatments, sorted cells that had gained PDGFRα⁺ expression (YFP⁺PDGFRα⁺ cells), but not YFP⁺PDGFRα⁻ cells, were positive for oil red staining (adipocyte), alkaline phosphatase staining (osteoblast), or collagen staining (fibroblast) (Figures 5F and 5G). In contrast, YFP⁺ cells, which were PDGFRα⁻CD31^{low/-} or PDGFRα⁻α7^{low/-}, did not show multipotency (not shown), indicative of a more differentiated fibrogenic state. These results suggest that the process of fibrogenic plasticity of endothelial and myogenic cells within dystrophic muscle, as with the plastic response to TGFβ in vitro (see Figure 1), involves multipotent progenitor cell intermediate states. Of note, in vivo interference with PDGFRα signaling with imatinib (a tyrosine kinase inhibitor), which has been shown to target PDGFRα-expressing mesenchymal progenitor cells (Ito et al., 2013; Uezumi et al., 2014), prevented the loss

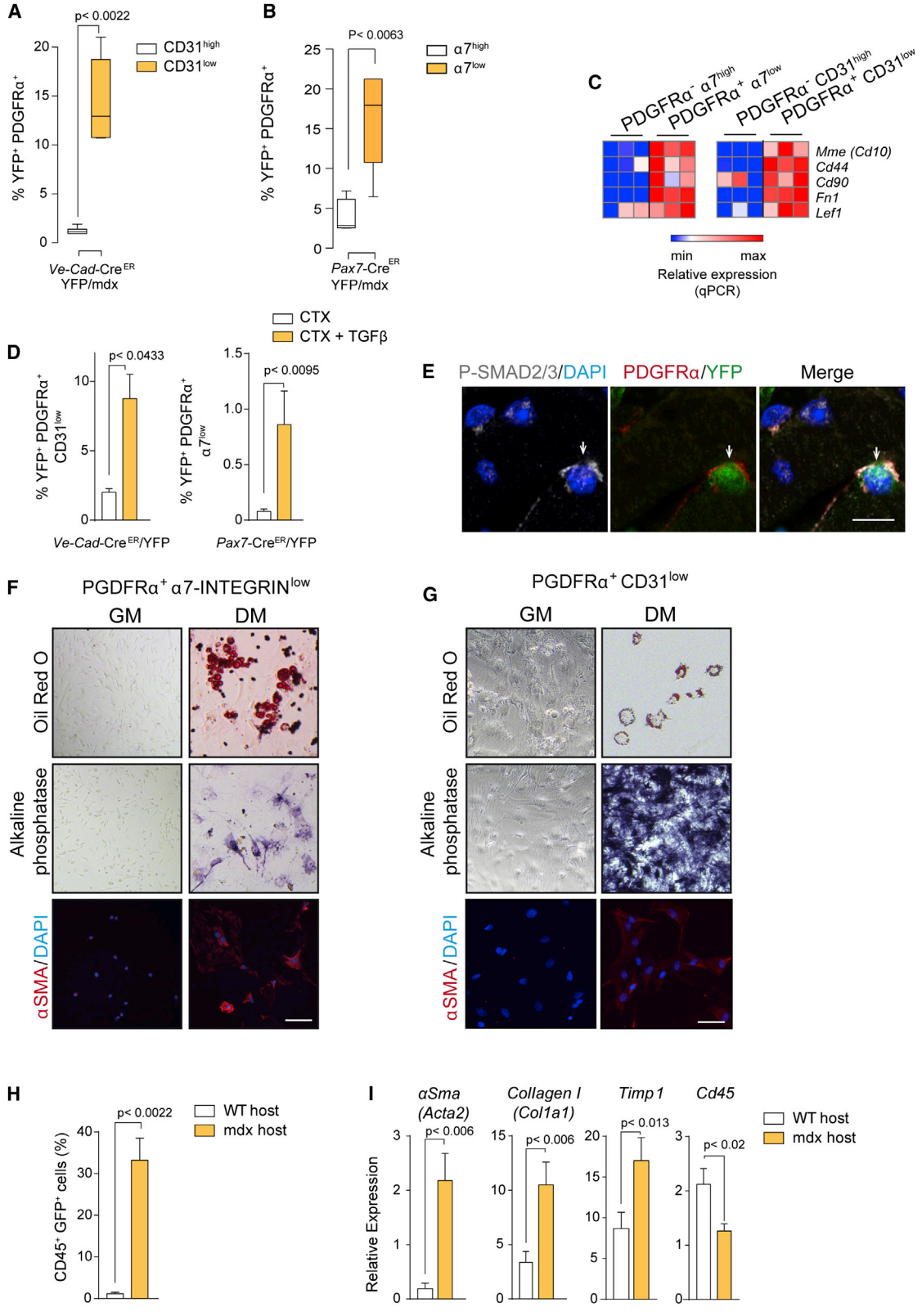
(B) Quantification of YFP⁺ cells that maintain or have reduced/lost α7-INTEGRIN expression in *Pax7-Cre^{ER}/YFP/mdx* (mdx) mice compared to *Pax7-Cre^{ER}/YFP* (WT) mice. Values are mean ± SEM; n = 6 animals for each group. Non-parametric Mann-Whitney U test was used for comparison.

(C and D) qRT-PCR of fibroblastic and myogenic markers in YFP⁺α7^{low/-} compared to YFP⁺α7^{high} cells from *Pax7-Cre^{ER}/YFP/mdx* muscle (C) and from CTX/TGFβ-injured *Pax7-Cre^{ER}/YFP* muscle (D). Values are mean ± SEM; n = 3 independent experiments (mice) for each group. Unpaired t test was used for comparison.

(E) Percentage of YFP⁺ cells that express myogenic and fibrogenic markers in single fibers from *Pax7-Cre/YFP/mdx* (mdx) mice compared to *Pax7-Cre/YFP* (WT) mice. Values are mean ± SEM; n = 5 animals each group. Non-parametric Mann-Whitney U test was used for comparison.

(F) Representative immunostaining shows YFP⁺ cells co-expressing fibrogenic markers in *Pax7-Cre^{ER}/YFP/mdx* mice. Scale bars, 25 μm.

(G) Myogenic potential of FACS-isolated YFP⁺α7^{high} cells from aged *Pax7-Cre/YFP/mdx* mice, compared to YFP⁺α7^{low/-} cells, after culture in DM for 4 days. The angiogenic potential of CD31^{high} versus CD31^{low/-} endothelial cells was similarly determined in angiogenic-promoting conditions (see Experimental Procedures). Scale bars, 50 μm.



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of cell identity in injured muscle in response to TGF β (Figure S5E).

Mesenchymal cells in the bone marrow are multipotent in nature. Since fibrocytes (defined as bone-marrow-derived cells expressing the hematopoietic marker *Cd45* and *Collagen I*) contribute to fibrosis in organs such as kidney, liver, lung, and heart (Duffield et al., 2013; Kisseleva and Brenner, 2012; Krenning et al., 2010), we hypothesized that fibrocytes also could contribute to fibroblast heterogeneity and fibrosis in dystrophic muscle. To test this possibility, we transplanted bone marrow from *Col1-GFP* reporter mice into young (3-month-old) *mdx/C57BL/6* mice, and the transplanted mice were aged for fibrosis development for 18 extra months. A significantly increased number of GFP⁺ cells was found in the muscle of transplanted dystrophic mice of 22 months of age (as an indication of collagen-producing cells derived from the donor bone marrow) compared to muscle of similarly transplanted non-dystrophic mice (Figure 5H). Consistent with this, compared to GFP⁺ cells from transplanted WT muscles, the FACS-isolated GFP⁺ cell population in aged dystrophic muscle showed reduced expression of the *Cd45* hematopoietic cell marker, while the expression of fibrogenic markers increased (Figure 5I), and this was in agreement with the detection of cells co-expressing fibrogenic and hematopoietic markers in advanced muscle disease stages (Figure S5F). As for endothelial and satellite cells, only 2%–3% of fibrocytes appeared to contribute to the overall population of collagen-expressing cells in dystrophic muscle (Figures S5A and S5B). Together, these results demonstrate the acquisition of fibrogenic traits by hematopoietic cells at advanced fibrotic states of muscular dystrophy, and further illustrate the existence of mesenchymal plasticity of distinct specialized cells in

dystrophic muscle, with deleterious consequences on disease progression.

As in dystrophic mice, we found that fibrosis was specifically induced in muscles of human DMD patients (compared to healthy individuals), being more prominent in affected patients between 6 and 8 years of age than in younger 2- to 4-year-old children (Figure 6A), and this correlated with disease severity and physical incapacitation (not shown). We also detected fibrogenic (TCF4⁺) cells in fibrotic muscle of DMD patients that increased with age (Figure 6A). In DMD muscles, we also identified fibrogenic cells co-expressing vWF (a marker of endothelial cells), CD56 (a marker of human satellite cells), and CD45 (a hematopoietic marker) (Figure 6B), demonstrating cell plasticity in the human pathology, as in *mdx* dystrophic mice (Figures S4C, S4G, and S5F; Figures 3, 4, and 5). Because active TGF β levels and signaling (P-SMAD2/3) also were increased in human DMD muscles, as fibrosis and disease severity progressed with age (Figure 6C), and because activated SMAD2/3 was specifically associated with cells expressing markers of fibrogenic and specialized cells in human and murine dystrophic muscle (Figures 6C and 6D; Figure S5D), these results, taken together, reinforce the idea of TGF β being a plasticity-promoting factor in DMD. Consistent with this, DMD muscles contained cells expressing the mesenchymal progenitor cell marker PDGFR α together with markers of endothelial, myogenic, and hematopoietic cells, respectively (Figure 6E), and PDGFR α -expressing cells that were double positive for P-SMAD2/3 and TCF4 (Figure 6D). Thus, in muscle of DMD patients, as in dystrophic mice, fibrogenic cells are a heterogeneous population, and part of these cells may arise from plastic events within the TGF β -enriched dystrophic milieu. Consistent herewith, human myoblasts also

Figure 5. Mesenchymal Progenitor Cells of Endothelial and Myogenic Origins from Dystrophic Muscle Exhibit Multipotent Capacity

(A and B) Percentage of double-positive YFP⁺ PDGFR α ⁺ cells from muscles of *Ve-Cad-Cre^{ER}/YFP/mdx* (that were CD31^{high} or CD31^{low}) mice (A) and *Pax7-Cre^{ER}/YFP/mdx* (that were α 7-INTEGRIN^{high} or α 7-iINTEGRIN^{low}) mice (B). Values are mean \pm SEM relative to age-matched lineage-tracing mice in non-dystrophic background; n = 6 animals for each group. Non-parametric Mann-Whitney U test was used for comparison.

(C) qRT-PCR of mesenchymal stem cell markers in YFP⁺/PDGFR α ⁺ cells with either α 7-INTEGRIN^{high} or α 7-INTEGRIN^{low} expression from mice in (B), or CD31^{high} or CD31^{low} expression from mice in (A, left). Values are mean \pm SEM; n = 3 independent experiments (mice) for each group.

(D) Percentage of YFP⁺ cells that express PDGFR α in CTX/TGF β -injured versus CTX-injured muscles from the indicated mouse genotypes. Values are mean \pm SEM; n = 4–6 for each group. Non-parametric Mann-Whitney U test was used for comparison.

(E) Representative immunostaining shows P-SMAD2/3 in PDGFR⁺/YFP⁺ cells in *Ve-Cad-Cre^{ER}/YFP/mdx* muscle. Scale bars, 10 μ m.

(F and G) Multipotency analysis of FACS-isolated YFP⁺PDGFR α ⁺ α 7^{low} and YFP⁺PDGFR α ⁺CD31^{low} from *Pax7-Cre/YFP/mdx* muscle (F) and *Ve-Cad-Cre^{ER}/YFP/mdx* muscle (G), cultured in adipogenic, osteogenic, and fibrogenic differentiation-promoting conditions (DM), compared to growth medium (GM)-cultured cells. Oil Red O, alkaline phosphatase, and α SMA staining are shown (n = 3 biological replicates). Scale bars, 50 μ m.

(H) Percentage of GFP⁺ cells in muscles of 22-month-old *mdx* mice, after transplantation of bone marrow from *Col1-GFP* mice. Values are mean \pm SEM; n = 3 animals for each group. Non-parametric Mann-Whitney U test was used for comparison.

(I) qRT-PCR for fibrogenic markers and CD45 of GFP⁺-sorted cells after bone marrow transplantation (H). Values are mean \pm SEM; n = 3 independent experiments (mice) for each group. Unpaired t test was used for comparison.

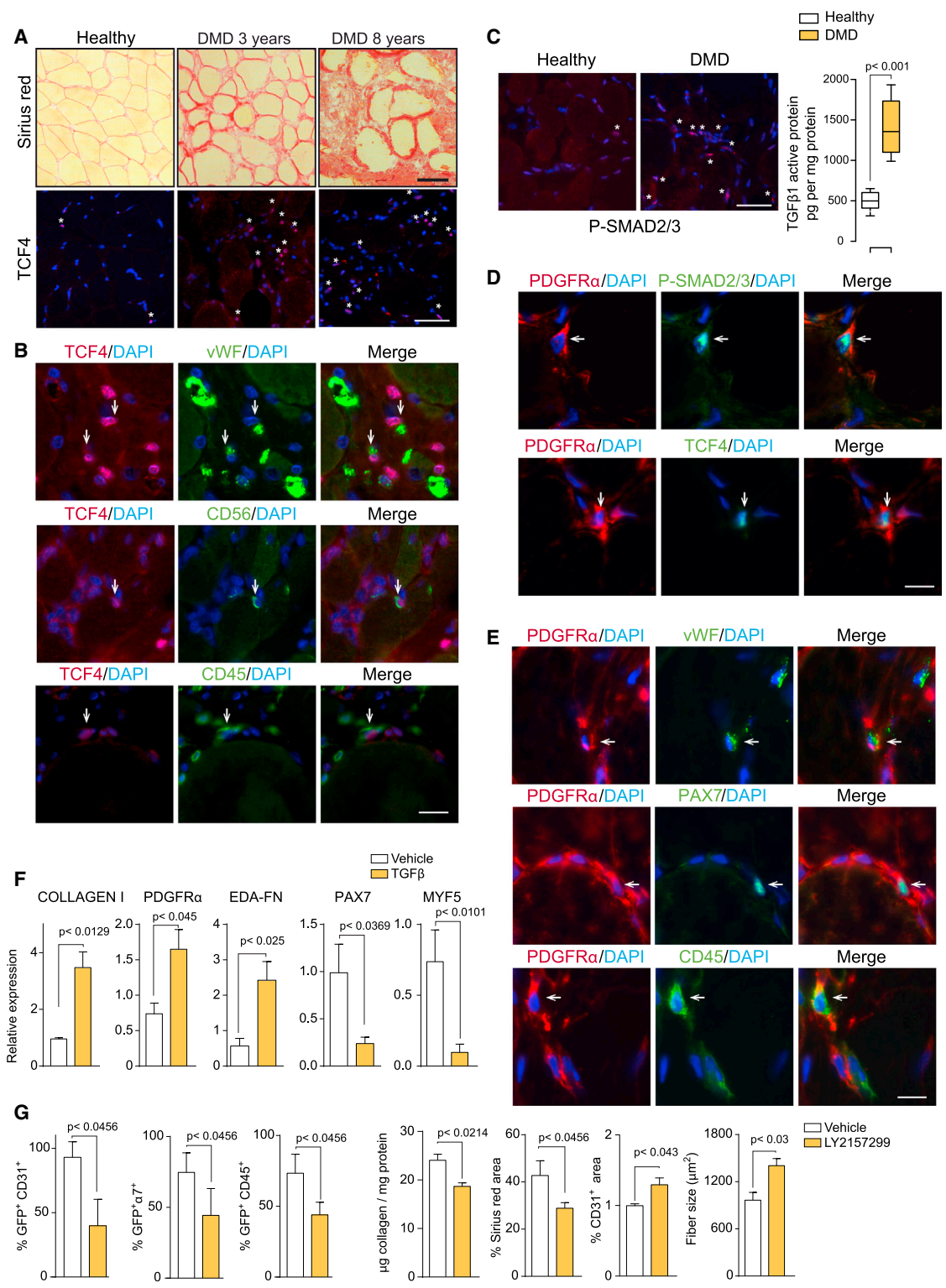


Figure 6. Fibrogenic Plasticity in Human Muscle of DMD Patients

(A) Representative Sirius Red and TCF4 staining in human muscle biopsies from healthy individuals and DMD patients 3 and 8 years of age are shown.

(legend continued on next page)



showed fibrogenic plasticity *in vitro* in response to TGF β treatment (Figure 6F). Moreover, *in vivo* inhibition of TGF β signaling, via the administration of LY2157299 (a specific inhibitor of the TGF β receptor type 1 kinase) (Zhou et al., 2011) in old dystrophic mdx mice, reduced the presence of collagen-producing cells co-expressing markers of the specialized lineages (Figure 6G), while reducing fibrosis and restoring dystrophic muscle regeneration and vascularization (Figure 6G). Altogether, these results unveil TGF β as a driver of fibrogenic cell plasticity both in human and mouse dystrophic and severely damaged muscle.

DISCUSSION

Our study provides insights into the mechanisms underlying the loss of regenerative potential and increasing fibrogenesis with age in DMD. We report that a proportion of specialized cells, which are critical for muscle regeneration, cannot maintain their identifying functions in aged dystrophic muscle of humans and mice, and acquire instead the capacity to produce matrix proteins. This is in agreement with a recent report from the Rando group (Biressi et al., 2014). Fibrogenesis in dystrophic muscle does not appear to be an all-or-nothing but rather a partial transition, as most cells share original as well as new fibroblastic traits, resembling the partial EMTs occurring in carcinosarcomas and fibrotic liver (Nieto, 2013; Sarrió et al., 2008; Zeisberg et al., 2007). This cellular plasticity, however, hampers muscle tissue repair potential. Mechanistically, we show that the loss of cell identity toward fibrogenesis in dystrophic muscle may involve the acquisition of mesenchymal traits, triggered by increasing TGF β as disease progresses. This link between skeletal muscle fibrogenesis and mesenchymal-like transitional states, resulting in the loss of regenerative potential, was a striking finding. Because adipose tissue also accumulates in muscle of DMD patients,

we postulated that the loss of identity of specialized cells concomitantly with the acquisition of mesenchymal-like (multipotent) characteristics might be a general feature for fibrogenesis as for adipogenesis in DMD, and by extension in other pathological conditions coursing with high TGF β signaling and tissue damage. Indeed, TGF β 2- or BMP4-induced EndMT into multipotent stem-like cells was proposed to be the origin of heterotopic cartilage and bone in individuals with fibrodysplasia ossificans progressiva (FOP) lesions (Medici et al., 2010). A distinct muscle-resident multipotent progenitor additionally was proposed as an origin for FOP, based on its osteogenic potential in response to BMP2 (Wosczyzna et al., 2012).

Recent studies have shown that, in addition to fibroblasts, tissue-resident FAPs and perivascular cells, which express PDGFR α , can differentiate to a fibroblastic fate and contribute to collagen accumulation in acutely injured muscle (Dulauroy et al., 2012; Joe et al., 2010; Uezumi et al., 2010). FAPs also accumulate in young dystrophic muscle prior to maximal fibrosis (Uezumi et al., 2011), being the main source of the fibrogenic progeny leading to collagen production at early dystrophy stages. Our results demonstrate that at advanced DMD stages there are additional reservoirs of fibrogenic cells derived from myogenic, endothelial, and hematopoietic cells. However, unlike organs like kidney, where a great proportion of the fibrotic cells arise from the bone marrow (and to a lesser extent from EndMT or EMT) (LeBleu et al., 2013), in dystrophic skeletal muscle, the net contribution of these cells to the actual population of collagen-producing cells (the key cells for fibrosis development) is modest. Instead, our results reveal that it is the loss of myogenic and endothelial cells' biochemical and phenotypic identities, through plastic mesenchymal transitions (characterized by the expression of PDGFR α), that causes severe deficits in myogenesis and angiogenesis, thus exacerbating the regenerative impairment in dystrophic muscle (see scheme in Figure 1). Therapeutically, a unifying model of loss of cell

(B) Representative picture of co-staining of TCF4 with endothelial, myogenic, and hematopoietic cell markers, respectively, in DMD muscles. Nuclei are stained with DAPI.

(C) Representative picture of P-SMAD2/3 staining and active TGF β protein levels in muscle biopsies from healthy individuals and DMD patients 6–8 years of age. Data correspond to the mean \pm SEM; $n = 10$ in each group. Non-parametric Mann-Whitney U test was used for comparison.

(D) Representative immunostaining shows human cell co-expressing PDGFR α and P-SMAD2/3 or TCF4 in DMD muscle.

(E) Representative immunostaining shows human cells expressing PDGFR α and vWF, PAX7, or CD45 in DMD muscle. Nuclei are stained with DAPI.

(F) Human myoblasts were treated with TGF β or vehicle for 6 days and analyzed for the expression of the indicated genes. Values are mean \pm SEM; $n = 3$ independent experiments for each group. Unpaired t test was used for comparison.

(G) Reduction of GFP $^+$ cells co-expressing CD31, α 7-INTEGRIN, or CD45 in adult *Col1I*-GFP/mdx muscle after treatment with the TGF β signaling inhibitor LY2157299 (or vehicle) for 1 month. Quantification shows fibrosis reduction and increased regeneration and vascularization. Data correspond to the mean \pm SEM; $n = 3$ for each group. Non-parametric Mann-Whitney U test was used for comparison. Scale bars, 50 μ m.



fate and acquisition of mesenchymal traits centered on the PDGFR α -expressing cell could be envisioned. How to restrict deleterious PDGFR α -dependent functions while preserving the beneficial ones is likely to be more challenging.

Our findings support the notion that, in chronic degenerative conditions, the ability of specialized cells (such as myogenic, endothelial, and inflammatory cells) to undergo mesenchymal transitions is inversely correlated to the degree of tissue regeneration, while facilitating fibrogenesis. This has implications for regenerative medicine, as our findings unravel a physiological form of plasticity that can be co-opted toward disease-associated tissue degeneration and aging.

EXPERIMENTAL PROCEDURES

Mice

Mdx mice were maintained in C57BL/10 or DBA/2 backgrounds. Lineage-tracing mice have been described previously as follows: *Cdh5*-Cre^{ER} (Wang et al., 2010), *Tie2*-Cre transgenic (Kisanuki et al., 2001), *Pax7*-Cre and *Pax7*-Cre^{ER} (Nishijo et al., 2009), R26R-EYFP (Srinivas et al., 2001), and *Col1a1*-3.6GFP (*Coll*-GFP) (Krempein et al., 1999). Lineage-tracing mice were intercrossed with C57BL/10 or DBA/2 dystrophic mdx mice (Fukada et al., 2010). When needed, Cre activity was induced by intraperitoneal injection (one injection per day for 4 days) with 5 mg/25 g body weight tamoxifen (TAM) (Sigma; 10 mg/ml in corn oil). Bone marrow transplantation experiments were performed as previously described (Perdiguero et al., 2011), using *Coll*-GFP mice as bone marrow donor and mdx/C57BL/10 at 3 months of age as recipient mice; mice were aged for 18 months for fibrosis development.

Induction of Muscle Regeneration and Fibrosis

Regeneration of skeletal muscle was induced by intramuscular injection of 50 μ l 10⁻⁵ M CTX (Latoxan). Muscles were collected at the indicated times on each set of experiments, which was usually 2 weeks after myotoxin injection. Contralateral control muscles were left un-injured. To induce a more severe fibrotic injury, tibialis anterior (TA) muscles of WT mice were subjected to laceration as previously described (Ardite et al., 2012). To exacerbate fibrotic muscle damage, 50 ng TGF β (recombinant TGF β 1 and TGF β 2, R&D Systems) was injected into previously injured (or dystrophic) TA and gastrocnemius muscle in a volume of 50 μ l PBS, as previously described (Pessina et al., 2014). For the inhibition of TGF β , LY2157299 (a specific inhibitor of the TGF β receptor type 1 kinase) (Zhou et al., 2011) was administered to old mdx mice (in the *Coll*-GFP background) for 30 days at a 25 mg/kg daily dose, and different parameters were analyzed thereafter. For the inhibition of PDGFR α signaling, imatinib (a tyrosine kinase inhibitor), which has been shown to target PDGFR α -expressing mesenchymal progenitor cells (Ito et al., 2013; Uezumi et al., 2014), was administered to 7-day-CTX-injured mice for an additional 7-day period (50 mg/kg daily), coinciding with the administration of TGF β , and analyzed subsequently.

Cell Culture and Differentiation

Primary myoblasts were obtained from mouse skeletal muscle, grown in Ham's F10 medium (BioWest) supplemented with 20% fetal bovine serum (FBS) and basic fibroblast growth factor (bFGF, 0.025 μ g ml⁻¹), as described previously (Perdiguero et al., 2011). Primary endothelial cells were grown in high-glucose DMEM (BioWest) supplemented with 10% FBS and 10 ng/ml vascular endothelial growth factor (VEGF), as described previously (Ieronimakis et al., 2008). Human myoblasts were purchased from Cook Myosite and cultured following the provided instructions. When indicated, cells were treated with TGF β 1 (myoblasts) or TGF β 2 (endothelial cells) (10 ng/ml). To induce differentiation into distinct lineages, cells were grown in StemXVivo osteogenic and adipogenic culture media (R&D Systems), after 4 days of TGF β treatment. Alkaline phosphatase staining to detect osteoblasts was performed with the alkaline phosphatase kit (Sigma-Aldrich) on cultures grown in osteogenic medium for 14 days. Oil red O (Sigma-Aldrich) staining to detect adipocytes was performed on cultures grown in adipogenic medium for 14 days. Alternatively, for fibrogenic differentiation, cells were cultured in TGF β -containing medium for an additional 7-day period.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism software using the nonparametric Mann-Whitney U test or unpaired t test for independent samples, with a confidence level of 95% being considered statistically significant. The results are expressed as mean \pm SEM. The number of samples analyzed per group is detailed in each figure.

ACCESSION NUMBERS

The accession number for the microarray data reported in this paper is GEO GSE67687.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2015.04.007>.

ACKNOWLEDGMENTS

We are indebted to V. Ruiz-Bonilla, V. Lukesova, S. Gutarra, M. Raya, B. Ampudia, and members of the Cell Biology Group for their contributions to this study. J. Martín-Caballero (PRBB Animal Facility), O. Fornas (CRG/UPF FACS unit), and CRG Genomic Unit. We also thank M. Reyes and N. Ieronimakis for help in isolation of muscle endothelial cells, S. Biressi and T. Rando for the generous offer to provide samples and for information exchange, C. Keller for *Pax7*-Cre lines, R.H. Adams and J.L. de la Pompa for *Ve-Cad*^{ER}-Cre and *Tie2*-Cre lines, D. Brenner for *Coll*-GFP reporter mice, and D. Medici for advice on cell plasticity studies. The authors acknowledge funding from the Ministry of Economy and Competitiveness (MINECO)-Spain (SAF2012-38547, PI13/02512, and PLE2009-0124), Association Française Myopathies (AFM), E-Rare, Fundació Marató TV3, Muscular



Dystrophy Association (MDA), European Commission Research and Innovation funding EU-FP7 (Myoage, Optistem, and Endostem), and Duchenne PP-NL. P.P. and Y.K. were partly supported by postdoctoral fellowships from AFM.

Received: December 24, 2014

Revised: April 17, 2015

Accepted: April 17, 2015

Published: May 14, 2015

REFERENCES

- Abou-Khalil, R., Mounier, R., and Chazaud, B. (2010). Regulation of myogenic stem cell behavior by vessel cells: the “ménage à trois” of satellite cells, periendothelial cells and endothelial cells. *Cell Cycle* 9, 892–896.
- Acuña, M.J., Pessina, P., Olguin, H., Cabrera, D., Vio, C.P., Bader, M., Muñoz-Canoves, P., Santos, R.A., Cabello-Verrugio, C., and Brandan, E. (2014). Restoration of muscle strength in dystrophic muscle by angiotensin-1-7 through inhibition of TGF- β signalling. *Hum. Mol. Genet.* 23, 1237–1249.
- Ardite, E., Perdiguero, E., Vidal, B., Gutarra, S., Serrano, A.L., and Muñoz-Cánoves, P. (2012). PAI-1-regulated miR-21 defines a novel age-associated fibrogenic pathway in muscular dystrophy. *J. Cell Biol.* 196, 163–175.
- Bioresi, S., Miyabara, E.H., Gopinath, S.D., Carlig, P.M., and Rando, T.A. (2014). A Wnt-TGF β 2 axis induces a fibrogenic program in muscle stem cells from dystrophic mice. *Sci. Transl. Med.* 6, 267ra176.
- Brack, A.S., Conboy, M.J., Roy, S., Lee, M., Kuo, C.J., Keller, C., and Rando, T.A. (2007). Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science* 317, 807–810.
- Chong, J.J., Reinecke, H., Iwata, M., Torok-Storb, B., Stempien-Otero, A., and Murry, C.E. (2013). Progenitor cells identified by PDGFR- α expression in the developing and diseased human heart. *Stem Cells Dev.* 22, 1932–1943.
- Duffield, J.S., Lupher, M., Thannickal, V.J., and Wynn, T.A. (2013). Host responses in tissue repair and fibrosis. *Annu. Rev. Pathol.* 8, 241–276.
- Dulauroy, S., Di Carlo, S.E., Langa, F., Eberl, G., and Peduto, L. (2012). Lineage tracing and genetic ablation of ADAM12(+) perivascular cells identify a major source of profibrotic cells during acute tissue injury. *Nat. Med.* 18, 1262–1270.
- Fukada, S., Morikawa, D., Yamamoto, Y., Yoshida, T., Sumie, N., Yamaguchi, M., Ito, T., Miyagoe-Suzuki, Y., Takeda, S., Tsujikawa, K., and Yamamoto, H. (2010). Genetic background affects properties of satellite cells and mdx phenotypes. *Am. J. Pathol.* 176, 2414–2424.
- Ieronimakis, N., Balasundaram, G., and Reyes, M. (2008). Direct isolation, culture and transplant of mouse skeletal muscle derived endothelial cells with angiogenic potential. *PLoS ONE* 3, e0001753.
- Ito, T., Ogawa, R., Uezumi, A., Ohtani, T., Watanabe, Y., Tsujikawa, K., Miyagoe-Suzuki, Y., Takeda, S., Yamamoto, H., and Fukada, S. (2013). Imatinib attenuates severe mouse dystrophy and inhibits proliferation and fibrosis-marker expression in muscle mesenchymal progenitors. *Neuromuscul. Disord.* 23, 349–356.
- Joe, A.W., Yi, L., Natarajan, A., Le Grand, F., So, L., Wang, J., Rudnicki, M.A., and Rossi, F.M. (2010). Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis. *Nat. Cell Biol.* 12, 153–163.
- Kharraz, Y., Guerra, J., Pessina, P., Serrano, A.L., and Muñoz-Cánoves, P. (2014). Understanding the process of fibrosis in Duchenne muscular dystrophy. *Biomed. Res. Int.* 2014, 965631.
- Kisanuki, Y.Y., Hammer, R.E., Miyazaki, J., Williams, S.C., Richardson, J.A., and Yanagisawa, M. (2001). Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev. Biol.* 230, 230–242.
- Kisseleva, T., and Brenner, D.A. (2012). The phenotypic fate and functional role for bone marrow-derived stem cells in liver fibrosis. *J. Hepatol.* 56, 965–972.
- Krempen, K., Grotkopp, D., Hall, K., Bache, A., Gillan, A., Rippe, R.A., Brenner, D.A., and Breindl, M. (1999). Far upstream regulatory elements enhance position-independent and uterus-specific expression of the murine α 1(I) collagen promoter in transgenic mice. *Gene Expr.* 8, 151–163.
- Krenning, G., Zeisberg, E.M., and Kalluri, R. (2010). The origin of fibroblasts and mechanism of cardiac fibrosis. *J. Cell. Physiol.* 225, 631–637.
- Kubo, H., Shimizu, M., Taya, Y., Kawamoto, T., Michida, M., Kaneko, E., Igarashi, A., Nishimura, M., Segoshi, K., Shimazu, Y., et al. (2009). Identification of mesenchymal stem cell (MSC)-transcription factors by microarray and knockdown analyses, and signature molecule-marked MSC in bone marrow by immunohistochemistry. *Genes Cells* 14, 407–424.
- Kumarswamy, R., Volkmann, I., Jazbutyte, V., Dangwal, S., Park, D.H., and Thum, T. (2012). Transforming growth factor- β -induced endothelial-to-mesenchymal transition is partly mediated by microRNA-21. *Arterioscler. Thromb. Vasc. Biol.* 32, 361–369.
- LeBleu, V.S., Taduri, G., O’Connell, J., Teng, Y., Cooke, V.G., Woda, C., Sugimoto, H., and Kalluri, R. (2013). Origin and function of myofibroblasts in kidney fibrosis. *Nat. Med.* 19, 1047–1053.
- Mann, C.J., Perdiguero, E., Kharraz, Y., Aguilar, S., Pessina, P., Serrano, A.L., and Muñoz-Cánoves, P. (2011). Aberrant repair and fibrosis development in skeletal muscle. *Skelet. Muscle* 1, 21.
- Medici, D., and Kalluri, R. (2012). Endothelial-mesenchymal transition and its contribution to the emergence of stem cell phenotype. *Semin. Cancer Biol.* 22, 379–384.
- Medici, D., Shore, E.M., Lounev, V.Y., Kaplan, F.S., Kalluri, R., and Olsen, B.R. (2010). Conversion of vascular endothelial cells into multipotent stem-like cells. *Nat. Med.* 16, 1400–1406.
- Mounier, R., Chrétien, F., and Chazaud, B. (2011). Blood vessels and the satellite cell niche. *Curr. Top. Dev. Biol.* 96, 121–138.
- Nieto, M.A. (2011). The ins and outs of the epithelial to mesenchymal transition in health and disease. *Annu. Rev. Cell Dev. Biol.* 27, 347–376.
- Nieto, M.A. (2013). Epithelial plasticity: a common theme in embryonic and cancer cells. *Science* 342, 1234850.



- Nieto, M.A., and Cano, A. (2012). The epithelial-mesenchymal transition under control: global programs to regulate epithelial plasticity. *Semin. Cancer Biol.* *22*, 361–368.
- Nishijo, K., Hosoyama, T., Bjornson, C.R., Schaffer, B.S., Prajapati, S.I., Bahadur, A.N., Hansen, M.S., Blandford, M.C., McCleish, A.T., Rubin, B.P., et al. (2009). Biomarker system for studying muscle, stem cells, and cancer in vivo. *FASEB J.* *23*, 2681–2690.
- Perdiguerro, E., Sousa-Victor, P., Ruiz-Bonilla, V., Jardí, M., Caelles, C., Serrano, A.L., and Muñoz-Cánoves, P. (2011). p38/MKP-1-regulated AKT coordinates macrophage transitions and resolution of inflammation during tissue repair. *J. Cell Biol.* *195*, 307–322.
- Pessina, P., Cabrera, D., Morales, M.G., Riquelme, C.A., Gutiérrez, J., Serrano, A.L., Brandan, E., and Muñoz-Cánoves, P. (2014). Novel and optimized strategies for inducing fibrosis in vivo: focus on Duchenne Muscular Dystrophy. *Skelet. Muscle* *4*, 7.
- Pinho, S., Lacombe, J., Hanoun, M., Mizoguchi, T., Bruns, I., Kuni-saki, Y., and Frenette, P.S. (2013). PDGFR α and CD51 mark human nestin+ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *J. Exp. Med.* *210*, 1351–1367.
- Sarrió, D., Rodríguez-Pinilla, S.M., Hardisson, D., Cano, A., Moreno-Bueno, G., and Palacios, J. (2008). Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. *Cancer Res.* *68*, 989–997.
- Serrano, A.L., Mann, C.J., Vidal, B., Ardite, E., Perdiguerro, E., and Muñoz-Cánoves, P. (2011). Cellular and molecular mechanisms regulating fibrosis in skeletal muscle repair and disease. *Curr. Top. Dev. Biol.* *96*, 167–201.
- Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev. Biol.* *1*, 4.
- Stedman, H.H., Sweeney, H.L., Shrager, J.B., Maguire, H.C., Panetier, R.A., Petrof, B., Narusawa, M., Leferovich, J.M., Sladky, J.T., and Kelly, A.M. (1991). The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. *Nature* *352*, 536–539.
- Uezumi, A., Fukada, S., Yamamoto, N., Takeda, S., and Tsuchida, K. (2010). Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle. *Nat. Cell Biol.* *12*, 143–152.
- Uezumi, A., Ito, T., Morikawa, D., Shimizu, N., Yoneda, T., Segawa, M., Yamaguchi, M., Ogawa, R., Matev, M.M., Miyagoe-Suzuki, Y., et al. (2011). Fibrosis and adipogenesis originate from a common mesenchymal progenitor in skeletal muscle. *J. Cell Sci.* *124*, 3654–3664.
- Uezumi, A., Fukada, S., Yamamoto, N., Ikemoto-Uezumi, M., Nakatani, M., Morita, M., Yamaguchi, A., Yamada, H., Nishino, I., Hamada, Y., and Tsuchida, K. (2014). Identification and characterization of PDGFR α + mesenchymal progenitors in human skeletal muscle. *Cell Death Dis.* *5*, e1186.
- Vidal, B., Serrano, A.L., Tjwa, M., Suelves, M., Ardite, E., De Mori, R., Baeza-Raja, B., Martínez de Lagrán, M., Lafuste, P., Ruiz-Bonilla, V., et al. (2008). Fibrinogen drives dystrophic muscle fibrosis via a TGFbeta/alternative macrophage activation pathway. *Genes Dev.* *22*, 1747–1752.
- Wallace, G.Q., and McNally, E.M. (2009). Mechanisms of muscle degeneration, regeneration, and repair in the muscular dystrophies. *Annu. Rev. Physiol.* *71*, 37–57.
- Wang, Z., Storb, R., Lee, D., Kushmerick, M.J., Chu, B., Berger, C., Arnett, A., Allen, J., Chamberlain, J.S., Riddell, S.R., and Tapscott, S.J. (2010). Immune responses to AAV in canine muscle monitored by cellular assays and noninvasive imaging. *Mol. Ther.* *18*, 617–624.
- Wosczyzna, M.N., Biswas, A.A., Cogswell, C.A., and Goldhamer, D.J. (2012). Multipotent progenitors resident in the skeletal muscle interstitium exhibit robust BMP-dependent osteogenic activity and mediate heterotopic ossification. *J. Bone Miner. Res.* *27*, 1004–1017.
- Zeisberg, M., and Kalluri, R. (2013). Cellular mechanisms of tissue fibrosis. 1. Common and organ-specific mechanisms associated with tissue fibrosis. *Am. J. Physiol. Cell Physiol.* *304*, C216–C225.
- Zeisberg, M., Yang, C., Martino, M., Duncan, M.B., Rieder, F., Tanjore, H., and Kalluri, R. (2007). Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *J. Biol. Chem.* *282*, 23337–23347.
- Zhou, L., McMahon, C., Bhagat, T., Alencar, C., Yu, Y., Fazzari, M., Sohal, D., Heuck, C., Gundabolu, K., Ng, C., et al. (2011). Reduced SMAD7 leads to overactivation of TGF-beta signaling in MDS that can be reversed by a specific inhibitor of TGF-beta receptor I kinase. *Cancer Res.* *71*, 955–963.
- Zordan, P., Rigamonti, E., Freudenberg, K., Conti, V., Azzoni, E., Rovere-Querini, P., and Brunelli, S. (2014). Macrophages commit postnatal endothelium-derived progenitors to angiogenesis and restrict endothelial to mesenchymal transition during muscle regeneration. *Cell Death Dis.* *5*, e1031.