Fibrosis and adipogenesis originate from a common mesenchymal progenitor in skeletal muscle

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Accepted 17 June 2011
Journal of Cell Science 124, 3654–3664

doi: 10.1242/jcs.086629

Summary

Accumulation of adipocytes and collagen type-I-producing cells (fibrosis) is observed in muscular dystrophies. The origin of these cells had been largely unknown, but recently we identified mesenchymal progenitors positive for platelet-derived growth factor receptor alpha (PDGFRα) as the origin of adipocytes in skeletal muscle. However, the origin of muscle fibrosis remains largely unknown. In this study, clonal analyses show that PDGFRα$^+$ cells also differentiate into collagen type-I-producing cells. In fact, PDGFRα$^+$ cells accumulated in fibrotic areas of the diaphragm in the *mdx* mouse, a model of Duchenne muscular dystrophy. Furthermore, mRNA of fibrosis markers was expressed exclusively in the PDGFRα$^+$ cell fraction in the *mdx* diaphragm. Importantly, TGF-β isoforms, known as potent profibrotic cytokines, induced expression of markers of fibrosis in PDGFRα$^+$ cells but not in myogenic cells. Transplantation studies revealed that fibrogenic PDGFRα$^+$ cells mainly derived from pre-existing PDGFRα$^+$ cells and that the contribution of PDGFRα$^-$ cells and circulating cells was limited. These results indicate that mesenchymal progenitors are the main origin of not only fat accumulation but also fibrosis in skeletal muscle.

Key words: Muscular dystrophy, PDGFRα, Satellite cell

Introduction

Muscle satellite cells are located beneath the basal lamina and begin to proliferate when muscle is damaged (Charge and Rudnicki, 2004). Although muscle satellite cells have great regenerative potential (Collins et al., 2005), some experimental and pathological conditions lead to impaired regeneration, with fat accumulation and/or fibrosis in skeletal muscle. Both Segawa and colleagues and Warren and colleagues reported the appearance of both fat accumulation and fibrosis in macrophage-suppressed mice (Segawa et al., 2008; Warren et al., 2005). Fat accumulation and fibrosis are also observed in mice lacking *Myf5*, a myogenic-determination gene (Gayraud-Morel et al., 2007). Furthermore, it is widely known that an increase in fatty and fibrous connective tissue is a hallmark of advanced Duchenne muscular dystrophy (DMD), which is caused by a mutation of the *dystrophin* gene (Carpenter and Karpati, 2001). It is considered that active pathogenic conditions, particularly fibrosis, not only exacerbate the loss of skeletal muscle function in DMD patients but also render cell and gene therapies for replacement of defective genes less feasible and effective. Therefore, suppression of the pathogenic progression is a crucial problem in treating patients by cell and gene therapies (Gargioli et al., 2008).

Fibrosis is characterized by excessive accumulation of extracellular matrix (ECM), and collagen type I is a major component of fibrotic ECM. The accumulated ECM replaces normal parenchymal tissue and can affect tissues and organ systems. In various tissues, fibrosis is generally induced by chronic injury, and the origin of the ECM-producing cells has been elucidated. Although it was originally considered that local interstitial cells of various tissues, called fibroblasts, were the main producers of ECM components, it has since been reported that the origins of ECM-producing cells are diverse and complex. It is well known that perivascular mesenchymal cells, called hepatic stellate cells, are the key fibrogenic cells in liver fibrosis (Friedman, 2000). In addition, Zeisberg et al. reported that adult hepatocytes contribute to liver fibrosis by means of an epithelial–mesenchymal transition (EMT) (Zeisberg et al., 2007b). EMT is also the main source of fibroblasts in kidney and lung fibrosis (Iwano et al., 2002; Kim et al., 2006). In addition, Iwano and colleagues adjudged that bone-marrow-derived circulating cells, named fibrocytes, could be another source of kidney fibrosis (Iwano et al., 2002). By contrast, Lin et al. showed that pericytes and perivascular fibroblasts are the primary source of collagen type-I-producing cells in the kidney (Lin et al., 2008). Moreover, Zeisberg and colleagues showed that adult coronary endothelial cells undergo endothelial–mesenchymal transition during cardiac fibrosis and contribute to cardiac fibrogenic cells (Zeisberg et al., 2007a).

Li and colleagues have reported that TGF-β, a profibrotic cytokine, in skeletal muscle induces the differentiation of C2C12 cells, a myogenic cell line, into fibrotic cells (Li et al., 2004). Alexakis et al. also indicated that collagen types I and III were expressed in primary myoblasts derived from mouse satellite

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cells (Alexakis et al., 2007). However, the origin of fibrosis in skeletal muscle has not been determined in vivo.

Recently, we revealed that skeletal-muscle-resident mesenchymal progenitors expressing PDGFRα (hereafter referred to as PDGFRα$^+$) contribute to ectopic fat formation in skeletal muscle (Uezumi et al., 2010). Joe et al. showed that this cell population also has the ability to differentiate into fibroblasts in vitro (Joe et al., 2010). In addition, using constitutively active PDGFRα knock-in mice, Olson et al. demonstrated that forced activation of PDGFRα signaling induced systemic fibrosis, including that of skeletal muscle (Olson and Soriano, 2009). These results imply that fibrosis and fat accumulation might be caused by a common PDGFRα$^+$ mesenchymal progenitor in skeletal muscle. To elucidate whether the PDGFRα$^+$ mesenchymal progenitors also contribute to muscle fibrosis, we performed clonal analysis and found that cells derived from PDGFRα$^+$ cells can differentiate into both adipocytes and collagen type-I-producing cells. Furthermore, both in vitro and in vivo experiments show that PDGFRα$^+$ cells exclusively express fibrosis markers, such as collagen types I and III, and connective tissue growth factor (CTGF). Importantly, TGF-β induced the expression of fibrosis markers in PDGFRα$^+$ cells, but not in myogenic cells. Finally, transplantation studies indicate that PDGFRα$^+$ cells are rarely derived from PDGFRα$^-$ or circulating cells. These findings reveal that skeletal muscle fibrosis originates from a common mesenchymal progenitor that contributes to fat accumulation and fibrosis in skeletal muscle under pathogenic conditions. These results might lead to the discovery of a new treatment for muscular dystrophy.

Results

A single PDGFRα$^+$ mesenchymal progenitor produces both adipocytes and collagen type-I-producing cells

To elucidate the contribution of PDGFRα$^+$ cells to fibrosis, we first fractionated whole mononucleated cells that had been isolated from injured skeletal muscles 3 days after cardiotoxin (CTX)

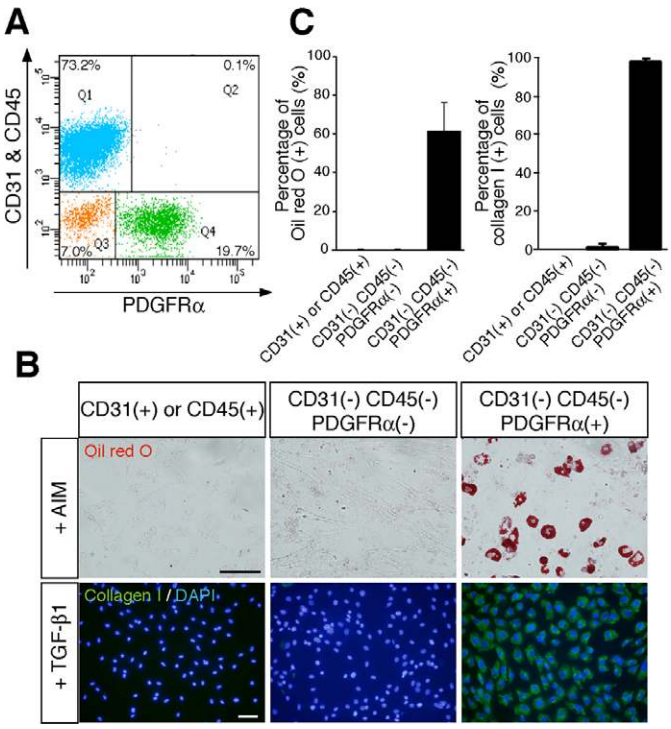

Fig. 1. Adipogenic and fibrogenic potentials of PDGFRα$^+$ cells derived from regenerating muscle. (**A**) Cells derived from regenerating limb muscle were analyzed for CD31, CD45 and PDGFRα expression by flow cytometry. (**B**) CD31$^+$ or CD45$^+$ cells, CD31$^-$ CD45$^-$ PDGFRα$^-$ cells and CD31$^-$ CD45$^-$ PDGFRα$^+$ cells cultured in adipogenesis induction medium (AIM) or DMEM containing 1 ng/ml TGF-β1 were stained with Oil red O or immunostained with antibody against collagen I. Nuclei were stained with DAPI (blue). Scale bars: 50 μm. (**C**) The adipogenic and fibrogenic differentiation potentials were evaluated by quantifying Oil red O-positive and collagen I-positive cells, respectively.

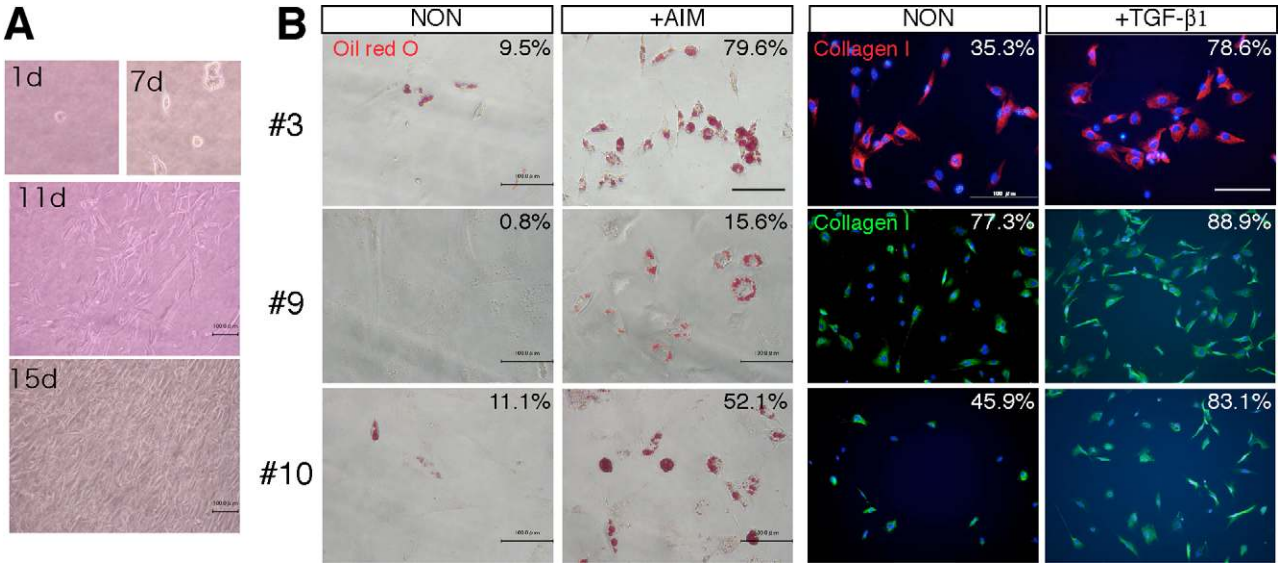

Fig. 2. Single PDGFRα$^+$ cells have diverse differentiation potentials. (**A**) A single PDGFRα$^+$ cell was cultured until a colony was formed (1–15 days). Scale bars: 100 μm. (**B**) Expanded cells derived from a single PDGFRα$^+$ cell were induced to become adipocytes (with AIM) or collagen-I-producing cells (with 1 ng/ml TGF-β1). Adipocytes and collagen-I-producing cells were detected using Oil red O staining and collagen I immunostaining, respectively. The numbers (3, 9, 10) are the identifiers of individual colonies derived from different single PDGFRα$^+$ cells. Scale bars: 100 μm. Non, negative control.

injection based on the expression of the following cell-surface markers: an endothelial marker – CD31, a pan-hematopoietic marker – CD45, and a mesenchymal progenitor marker – PDGFRα (Fig. 1A). Each cell fraction was then additionally cultured in adipocyte-inducing medium (AIM) or in medium containing TGF-β1 (a potent profibrotic cytokine). Similar to the results of cultures of PDGFRα$^+$ cells derived from intact skeletal muscle (Uezumi et al., 2010), only the PDGFRα$^+$ cells differentiated into adipocytes (Fig. 1B,C). In addition, in the presence of TGF-β1, collagen type-I-producing cells also appeared exclusively in the PDGFRα$^+$ fraction (Fig. 1B,C). More than 90% of PDGFRα$^+$ cells derived from intact muscle exhibit adipogenic potential. By contrast, in PDGFRα$^+$ cells derived from injured muscle, the frequency of adipogenic differentiation was ~60%. These results suggest that some PDGFRα$^+$ cells lose their adipogenic potential during the regeneration process.

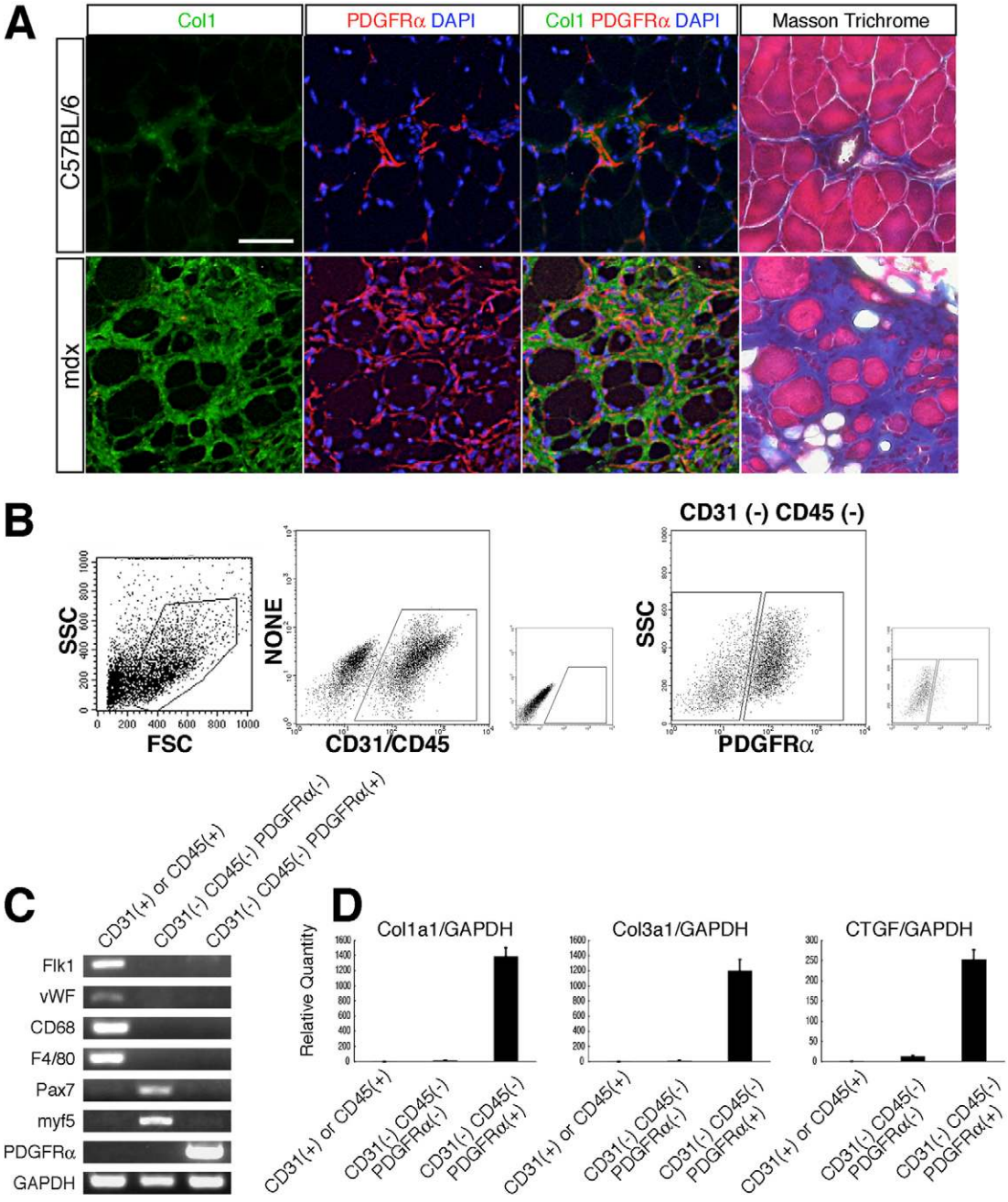

Fig. 3. PDGFRα$^+$ cells accumulate, and fibrosis markers are detected, in only the PDGFRα$^+$ CD31$^-$ CD45$^-$ fraction of dystrophic skeletal muscle. (**A**) Sections of diaphragms of normal C57BL/6 and *mdx* mice were stained with antibodies against collagen type I (green) and PDGFRα (red). Nuclei were stained with DAPI (blue). Serial sections were stained with Masson trichrome. Scale bar: 50 μm. (**B**) Whole diaphragm-derived cells were analyzed for CD31, CD45 and PDGFRα expression by flow cytometry. The CD31$^-$ CD45$^-$ fraction was separated into PDGFRα$^+$ or PDGFRα$^-$ fractions, as indicated. Positive and negative gates were set by analyzing a control sample stained only with secondary reagent or isotype antibody (shown on the right). Side scatter (SSC) and forward scatter (FSC) indicate the cell granularity and cell size, respectively. (**C**) RT-PCR analysis of lineage markers in the three freshly isolated populations indicated. (**D**) Transcripts of fibrosis-related genes encoding Col1a1, Col3a1 and CTGF were quantified by qRT-PCR.

We next expanded colonies derived from a single PDGFRα$^+$ cell (Fig. 2A) and treated clonally expanded cells with either AIM or TGF-β1. As shown in Fig. 2B, Oil-red-O-positive and collagen type-I-producing cells originated from a single PDGFRα$^+$ cell. Thirteen out of 14 clones showed this bi-potentiality. Although TGF-β1 treatment promoted collagen I expression, non-treated cells also contained many collagen type-I-positive cells. Clonal analysis requires 4–8 weeks, and PDGFRα$^+$ cells can differentiate into collagen type-I-positive cells under the condition of long-term culture. These results suggest the possibility that adipocytes and collagen type-I-producing cells are derived from a common multipotent mesenchymal progenitor.

PDGFRα$^+$ cells accumulate in fibrotic areas and express fibrosis-related molecules in the *mdx* diaphragm

It is widely known that the *mdx* mouse, a mouse model of DMD, exhibits fibrosis in the diaphragm similar to that of DMD patients (Stedman et al., 1991). To examine the existence and localization of PDGFRα$^+$ cells in a dystrophic environment, sections of *mdx* diaphragm were stained with antibodies against collagen type I and PDGFRα. In normal mice, excessive deposition of collagen type I or ECM is rarely observed, and only a few PDGFRα$^+$ cells are located around blood vessels or in interstitial areas (Fig. 3A). By contrast, an excessive accumulation of collagen type I was observed in the diaphragm of *mdx*. Masson trichrome staining also indicated that ECM was accumulated in the *mdx* diaphragm (Fig. 3A). Intriguingly, many PDGFRα$^+$ cells had accumulated in areas where collagen type I was deposited.

We next examined whether PDGFRα$^+$ cells that have accumulated in a fibrotic area indeed contribute to expression of fibrosis-related molecules in vivo. CD31$^+$ or CD45$^+$, CD31$^-$ CD45$^-$ PDGFRα$^-$ and CD31$^-$ CD45$^-$ PDGFRα$^+$ cell fractions were freshly isolated from diaphragms of 20-week-old *mdx* mice using fluorescence-activated cell sorting (FACS) (Fig. 3B). The CD31$^+$ or CD45$^+$ cell fraction includes endothelial and hematopoietic cells, and the CD31$^-$ CD45$^-$ PDGFRα$^-$ cell fraction contains mainly myogenic cells. The purity of each sorted population was confirmed by analyzing the expression of endothelial, hematopoietic and myogenic genes (Fig. 3C). We then

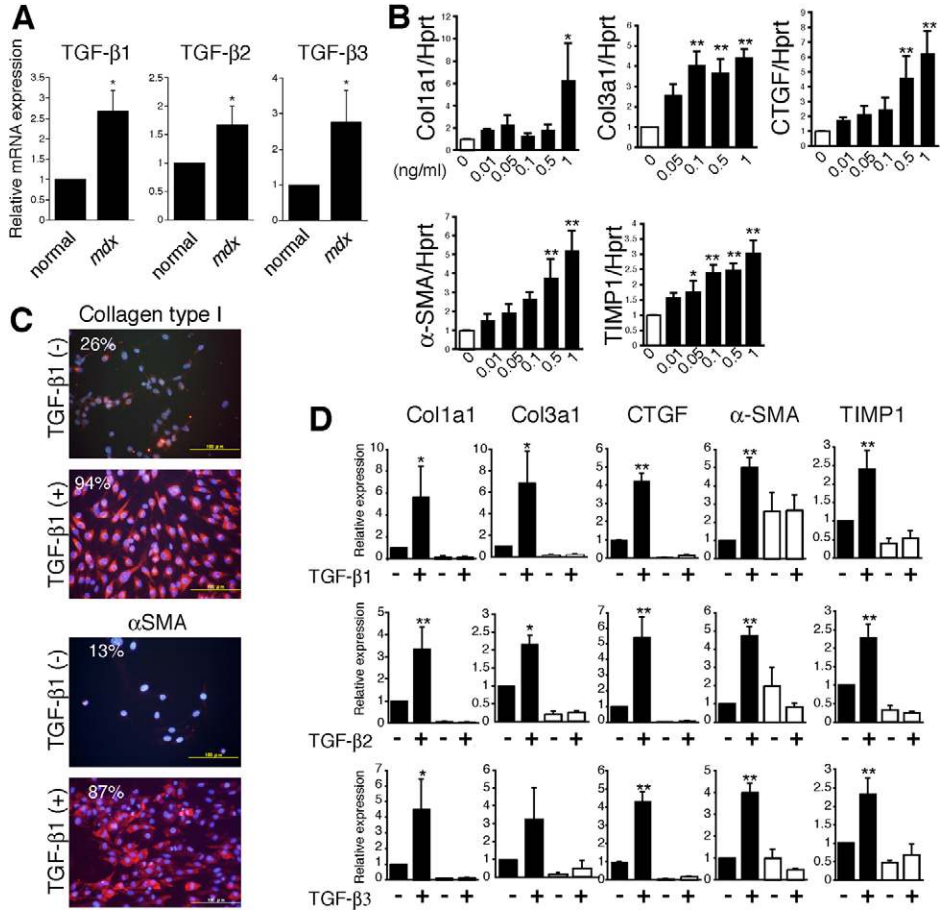

Fig. 4. TGF-β exclusively induces expression of fibrosis markers in PDGFRα$^+$ cells. (**A**) The expression of TGF-β isoforms in diaphragms of normal (C57BL/6) and *mdx* mice was evaluated by qRT-PCR. *P<0.05: indicates experimental pairs where differences between the compared values were statistically significant. (**B**) Transcripts of fibrosis-associated molecules in PDGFRα$^+$ cells cultured for 3 (Col1a1, CTGF, α-SMA and TIMP1) or 5 days (Col3a1) with 0–1 ng/ml TGF-β1 were quantified by qRT-PCR. *P<0.05 and **P<0.01: indicate where the values were significantly different from those of PDGFRα$^+$ cells that were not stimulated with TGF-β1. (**C**) PDGFRα$^+$ cells were cultured with or without TGF-β1 (1 ng/ml) and immunostained for collagen type I (red) or α-SMA (red). Nuclei were stained with DAPI (blue). Scale bars: 100 μm. (**D**) PDGFRα$^+$ cells (black bars) and primary myoblasts (white bars) were cultured with or without TGF-β (+ and − below the histograms indicate the presence and absence of TGF-β, respectively) and analyzed for mRNA expression of fibrosis markers by qRT-PCR. PDGFRα$^+$ cells and primary myoblasts were treated with 1 ng/ml and 5 ng/ml TGF-β isoforms, respectively. *P<0.05 and **P<0.01: indicate where the values were significantly different from those of PDGFRα$^+$ cells that were not stimulated with TGF-β1.

quantified the expression of fibrogenic genes using quantitative real-time PCR (qRT-PCR). As shown in Fig. 3D, PDGFRα$^+$ cells exclusively expressed collagens and CTGF. These results indicate that PDGFRα$^+$ cells are the main source of fibrosis-related molecules in dystrophic skeletal muscle.

TGF-β induces expression of fibrosis markers in PDGFRα$^+$ cells

TGF-β induces expression of fibrosis-related molecules, including collagen type I, in many cell types. It is also reported that TGF-β is highly expressed in DMD (Bernasconi et al., 1995). Furthermore, the expression of TGF-β isoforms was significantly upregulated in the *mdx* diaphragm where fibrosis is prominent (Fig. 4A). Therefore, in order to investigate whether TGF-β promotes the expressions of fibrosis-related molecules in PDGFRα$^+$ cells, PDGFRα$^+$ cells derived from the regenerating muscles 3 days after CTX injection were isolated, and, after a 3-day culture, stimulated with TGF-β1. For analysis of Col1a1, CTGF, alpha-smooth muscle actin (α-SMA) and tissue inhibitor of metalloprotease (TIMP1) expression, cells were treated with TGF-β isoforms for three days. The expression of Col3a1 was not increased at day 3, although it was at day 5 after treatment with the TGF-β isoforms. Therefore, the following experiments were performed at 3 days (Col1a1, CTGF, α-SMA and TIMP1) or 5 days (Col3a1) after stimulation with TGF-β isoforms.

As shown in Fig. 4B, we observed dose-dependent expression of Col1a1, Col3a1, CTGF, α-SMA and TIMP1 in PDGFRα$^+$ cells treated with TGF-β1. TGF-β2 and -β3 (TGF-β isoforms) also induced expression of these fibrosis-related molecules in PDGFRα$^+$ cells in a dose-dependent manner (data not shown).

We next examined the protein expression of collagen type I and α-SMA in PDGFRα$^+$ cells with or without TGF-β1 stimulation. As observed in Fig. 1, the percentage of collagen type-I-positive cells was increased in TGF-β1-stimulated PDGFRα$^+$ cells. Immunostaining of α-SMA also showed similar results (Fig. 4C).

Several studies have indicated that myogenic cells produce fibrosis markers such as collagens type I and III and CTGF (Alexakis et al., 2007; Li et al., 2004). Therefore, we next compared the gene expression of fibrosis-related molecules in PDGFRα$^+$ cells and primary myoblasts after stimulation with TGF-β1–TGF-β3. The concentration of TGF-β was determined as shown in Fig. 4B and supplementary material Fig. S1, and then PDGFRα$^+$ cells and primary myoblasts were treated with 1 ng/ml and 5 ng/ml of the TGF-β isoforms, respectively. As shown in Fig. 4D, the expression levels of fibrosis markers in PDGFRα$^+$ cells were much higher than those in myoblasts regardless of the TGF-β isoform tested.

We also compared the gene expression of fibrosis-related molecules between PDGFRα$^+$ cells and myogenic cells in vivo. PDGFRα$^+$ cells and myogenic cells were isolated from *mdx* diaphragms by FACS, and microarray analysis was then performed (Fig. 5A). This analysis identified 386 genes that were highly expressed in PDGFRα$^+$ cells (supplementary material Table S1) and 237 genes that were highly expressed in myogenic cells (supplementary material Table S2). We analyzed the 386 PDGFRα$^+$ cell-enriched genes based on gene ontology (GO) annotations or pathways. These analyses revealed that several GO categories and pathways were over-represented in PDGFRα$^+$ cells (supplementary material Tables S3, S4). Intriguingly, many ECM-related genes were found to be included in these over-represented GO categories and pathways. Because excess ECM production is a defining feature of fibrosis-initiating cells, we confirmed the expression of several ECM-related genes by RT-PCR. As shown in Fig. 5B, all ECM-related genes examined were highly enriched in PDGFRα$^+$ cells but not in myogenic cells. Together, these results further support the notion that the excess ECM deposition occurring in muscle fibrosis is attributable to PDGFRα$^+$ cells rather than to myogenic cells.

PDGFRα$^+$ cells accumulate in fibrotic areas

To examine the contribution of PDGFRα$^+$ cells to skeletal muscle fibrosis in vivo more directly, three cell types were transplanted into fibrotic muscle tissue. To induce impaired regeneration with fibrosis, the recipient mice were exposed to gamma radiation after intramuscular injection of CTX (supplementary material Fig. S2). This treatment led to severe fibrosis. Using this model, three cell populations from GFP-transgenic (Tg) mice were injected into the irradiated muscles. PDGFRα$^+$ cells (1.5×10^5 cells) and PDGFRα$^-$ cells (5×10^5 cells) were freshly isolated from intact skeletal muscle. Primary myoblasts were obtained using an SM/C-2.6 monoclonal antibody (Fukada et al., 2004). As shown in Fig. 6A,

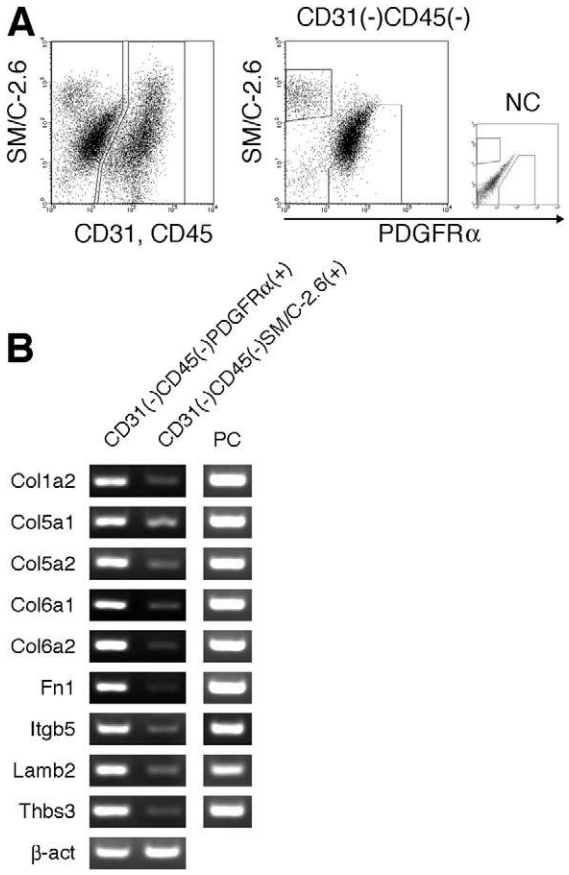

Fig. 5. Microarray analysis reveals fibrogenic feature of PDGFRα$^+$ cells. (**A**) *mdx*-diaphragm-derived cells were analyzed for the expression of CD31, CD45, SM/C-2.6 and PDGFRα. Positive gates were set by analyzing a negative-control (NC) sample stained only with secondary reagents. (**B**) Expression of ECM-related genes in CD31$^-$ CD45$^-$ PDGFRα$^+$ cells and CD31$^-$ CD45$^-$ SM/C-2.6$^+$ cells. RNA was extracted from freshly isolated cells immediately after cell sorting and RT-PCR was performed. RNA extracted from a whole embryo on embryonic day 13.5 was used as a positive control (PC).

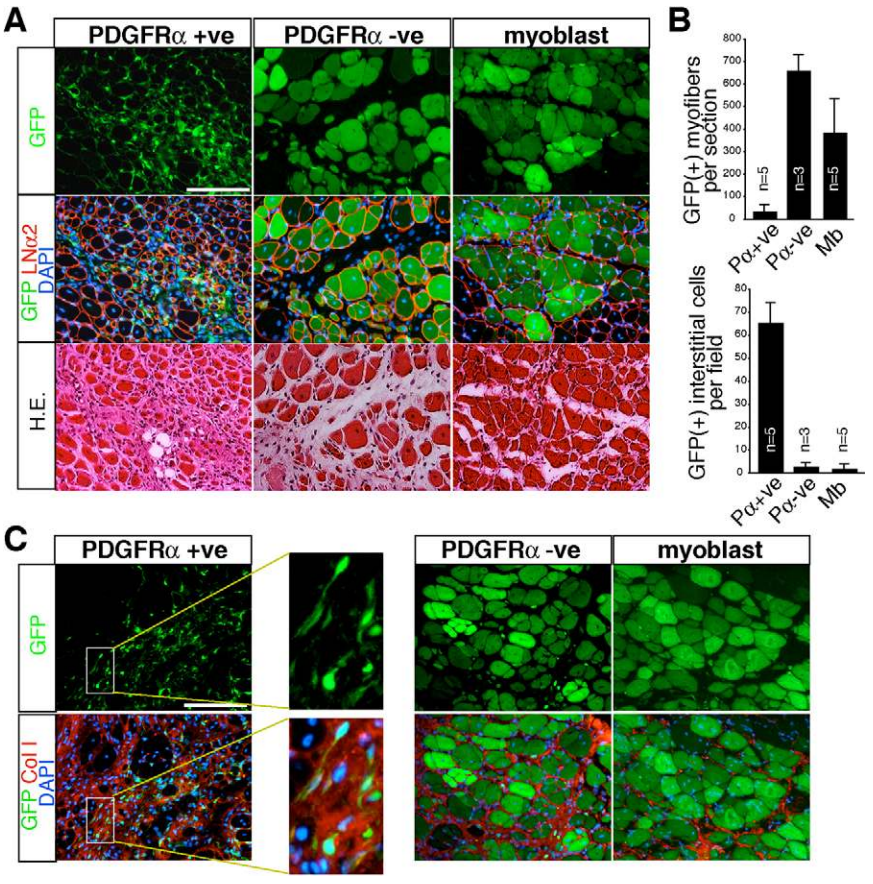

Fig. 6. PDGFRα$^+$ cells accumulate in areas of interstitial fibrosis in vivo. (**A**) PDGFRα$^+$ (Pα+ve) cells, PDGFRα$^-$ (Pα-ve) cells or myoblasts (Mb) isolated from muscles of GFP-Tg mice were injected into tibialis anterior (TA) muscles just after irradiation. Two weeks after transplantation, the TA muscles were analyzed for expression of laminin α2 (LNα2; red) and GFP (green) (middle and top). The same fields were observed after hematoxylin and eosin staining (H&E, bottom). Scale bar: 100 μm. (**B**) The histograms show the numbers of GFP$^+$ myofibers per section and GFP$^+$ interstitial mononuclear cells per field. The numbers of mice used in each study are shown against each column. (**C**) TA muscle sections were immunostained for collagen type I (red). GFP (green) was observed directly. DAPI (blue) was used to counterstain nuclei. Insets show high-magnification images. Scale bar: 100 μm.

GFP$^+$ cells were located in the interstitial spaces of muscles transplanted with PDGFRα$^+$ cells. The other cells – myoblasts and PDGFRα$^-$ cells – produced many GFP$^+$ myofibers, but the cells were rarely located in the interstitial spaces (Fig. 6A,B). In addition, transplanted PDGFRα$^+$ cells were located in fibrotic areas where collagen type I had accumulated, but PDGFRα$^-$ cell-derived or myoblast-derived cells were rarely observed there (Fig. 6C).

PDGFRα$^+$ cells are derived mainly from pre-existing PDGFRα$^+$ cells and rarely from PDGFRα$^-$ or circulating cells

To elucidate the origin of PDGFRα$^+$ cells in the fibrotic environment, we examined the expression of PDGFRα in GFP$^+$ cells. As shown in Fig. 7A, most of the transplanted PDGFRα$^+$ cells retained PDGFRα expression, whereas only a few GFP and PDGFRα double-positive cells were detected in PDGFRα$^-$-transplanted or myoblast-transplanted muscles. We also investigated whether circulating cells contribute to the PDGFRα$^+$ cell pool under the fibrotic condition. After transplantation of GFP-Tg mouse-derived bone marrow (BM), the diaphragms of recipient *mdx* mice were stained with antibodies against GFP, CD45 and PDGFRα. As shown in Fig. 7B, many CD45$^+$ cells – hematopoietic cells – expressed GFP. However, we detected very few GFP and PDGFRα double-positive cells. These results strongly suggested that fibrogenic PDGFRα$^+$ cells arise mainly from muscle-resident cells that expressed PDGFRα before fibrosis occurred and that the contribution of PDGFRα$^-$ cells, including satellite cells and BM-derived cells, to muscular fibrosis was very limited.

PDGFRα ligand promotes proliferation and expression of fibrosis markers in PDGFRα$^+$ cells

Finally, we examined the effects of PDGF–PDGFRα signaling on the proliferation of, and the expression of fibrosis markers by, PDGFRα$^+$ cells. Because serum contains PDGFRα ligands (Antoniades et al., 1979), the following experiments were performed in a serum-free medium. As shown in Fig. 8A, both TGF-β (1 ng/ml) and PDGF-AA (10 ng/ml) promoted the proliferation of PDGFRα$^+$ cells without a synergistic effect. An anti-PDGFRα–Fc chimera completely inhibited the effect of PDGF-AA. In addition, PDGF-AA (50 ng/ml) also promoted the expression of three fibrosis marker genes (encoding TIMP1, α-SMA and Col3a1), and an anti-PDGFRα–Fc chimera suppressed the expression of these genes in PDGFRα$^+$ cells. The levels of expression of TIMP1 and α-SMA were observed at 6 hours after PDGF-AA treatment. By contrast, expression of Col3a1 was observed at 48 hours. In serum-free conditions, TGF-β1 also induced each fibrosis molecule at the same time, but a synergistic effect of TGF-β and PDGF-AA was not observed in PDGFRα$^+$ cells (Fig. 8C). These results suggest that PDGF–PDGFRα signaling also plays a role in the expression of fibrosis markers and that TGF-β and PDGF use different signaling mechanisms to induce fibrosis.

Discussion

Mesenchymal progenitors or stem cells, and fibroblasts

It was originally believed that local interstitial cells, called ‘fibroblasts’, were the origin of ECM accumulation in fibrosis. However, many recent studies have indicated that the origins of

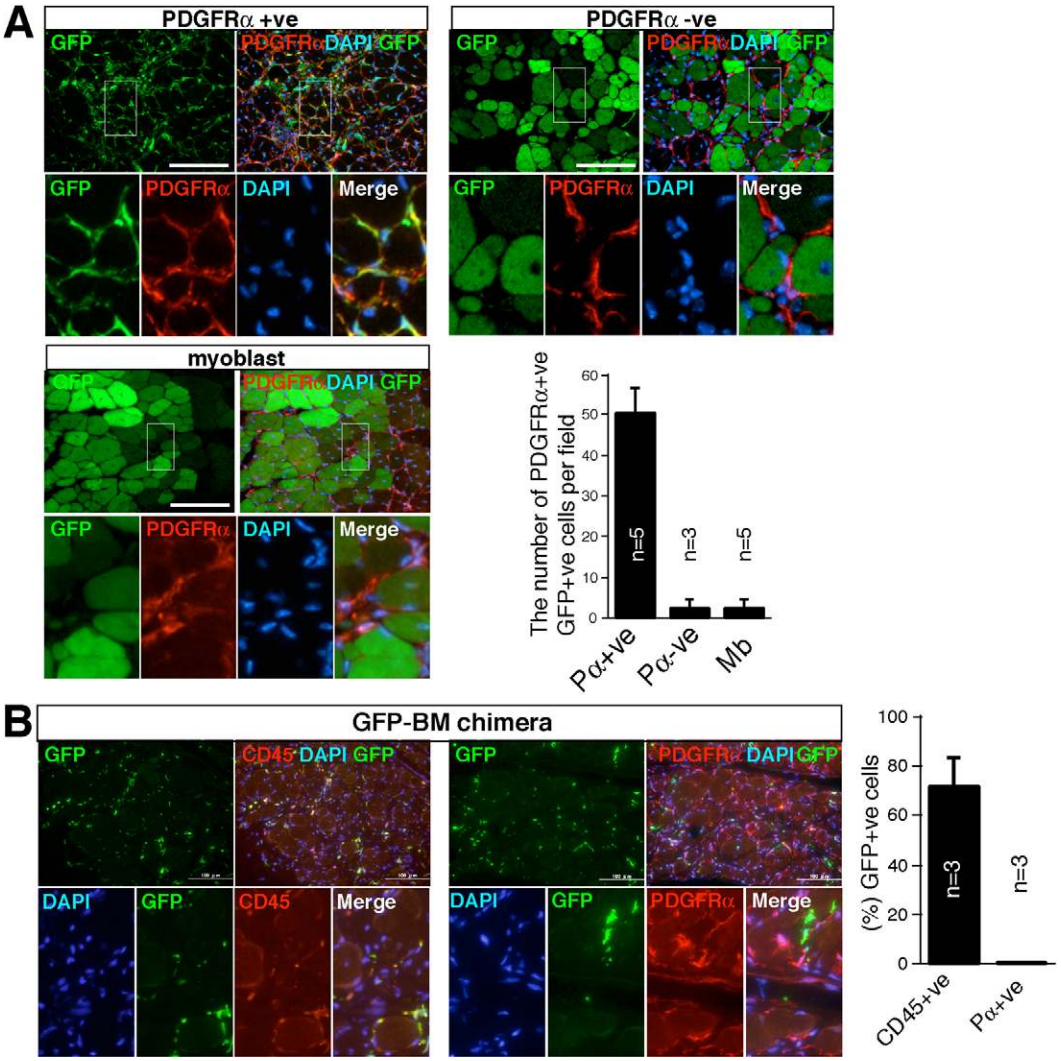

Fig. 7. Only a few PDGFRα$^+$ cells are derived from myogenic cells and circulating cells in vivo. (**A**) TA muscles transplanted with PDGFRα$^+$ cells, PDGFRα$^-$ cells or myoblasts (Mb) were immunostained with antibodies against PDGFRα (red) and GFP (green). The histogram shows the number of PDGFRα$^+$ GFP$^+$ cells. (**B**) The diaphragms of bone marrow (BM) chimera *mdx* mice were immunostained for CD45 (red) and GFP (green) (left side) and stained with antibodies against PDGFRα (red) and GFP (green) (right side). DAPI (blue) was used to counterstain nuclei. Scale bar: 100 μm. CD45$^+$ or PDGFRα$^+$ cells derived from circulating cells were evaluated by quantifying the percentage of CD45$^+$ GFP$^+$ cells and PDGFRα$^+$ (Pα+ve) GFP$^+$ cells. The numbers of mice used in each study are shown against each column.

ECM-producing cells in multiple organs and tissues are diverse and complex (Friedman, 2000; Iwano et al., 2002; Kim et al., 2006; Lin et al., 2008; Zeisberg et al., 2007b). Fibroblasts are easily obtained from various tissues and organs, and the morphology of fibroblasts resembles that of mesenchymal progenitors or stem cells. However, unlike mesenchymal progenitors or stem cells, it is thought that fibroblasts cannot differentiate into another mesenchymal lineage (Pittenger et al., 1999). Intriguingly, Sudo and colleagues recently reported that primary fibroblast-like cells derived from various human tissues were able to differentiate into at least one mesenchymal lineage, including osteoblasts, chondrocytes and adipocytes (Sudo et al., 2007). That study suggested that cell populations in various tissues originally considered to be fibroblasts contain mesenchymal progenitors or stem cells. In skeletal muscle, we recently identified mesenchymal progenitors that can differentiate into adipocytes, both in vitro and in vivo, using PDGFRα as a positive marker (Uezumi et al., 2010). In the present study, we showed that these PDGFRα$^+$ mesenchymal progenitors could also differentiate into ECM-producing cells. We cannot exclude the possibility that the PDGFRα$^+$ cell population contains fibroblasts, but our clonal analyses indicated that mesenchymal progenitors possess the multipotency to differentiate into both collagen type-I-producing cells and adipocytes. Recently, Morikawa and colleagues reported the prospective isolation of multipotent mesenchymal stem cells in murine bone marrow (Morikawa et al., 2009). They reported that bone-marrow-derived multipotent mesenchymal stem cells were present in the PDGFRα and Sca-1 double-positive fraction. PDGFRα$^+$ cells in skeletal muscle also coexpressed Sca-1 (Joe et al., 2010; Uezumi et al., 2010). Although the relationship between bone marrow and skeletal muscle mesenchymal progenitors is still unclear, our bone marrow transplantation study suggests that the contribution of bone marrow mesenchymal stem cells to muscle mesenchymal progenitors is very low.

Roles of TGF-β and PDGF signaling

TGF-β is one of the profibrotic cytokines that induce the expression of fibrosis-related molecules in various tissues. Bernasconi et al. reported a correlation between higher TGF-β expression and skeletal muscle fibrosis in DMD patients (Bernasconi et al., 1995). Zhou and colleagues showed that TGF-β1, -β2 and -β3 and their receptors are upregulated in *mdx*

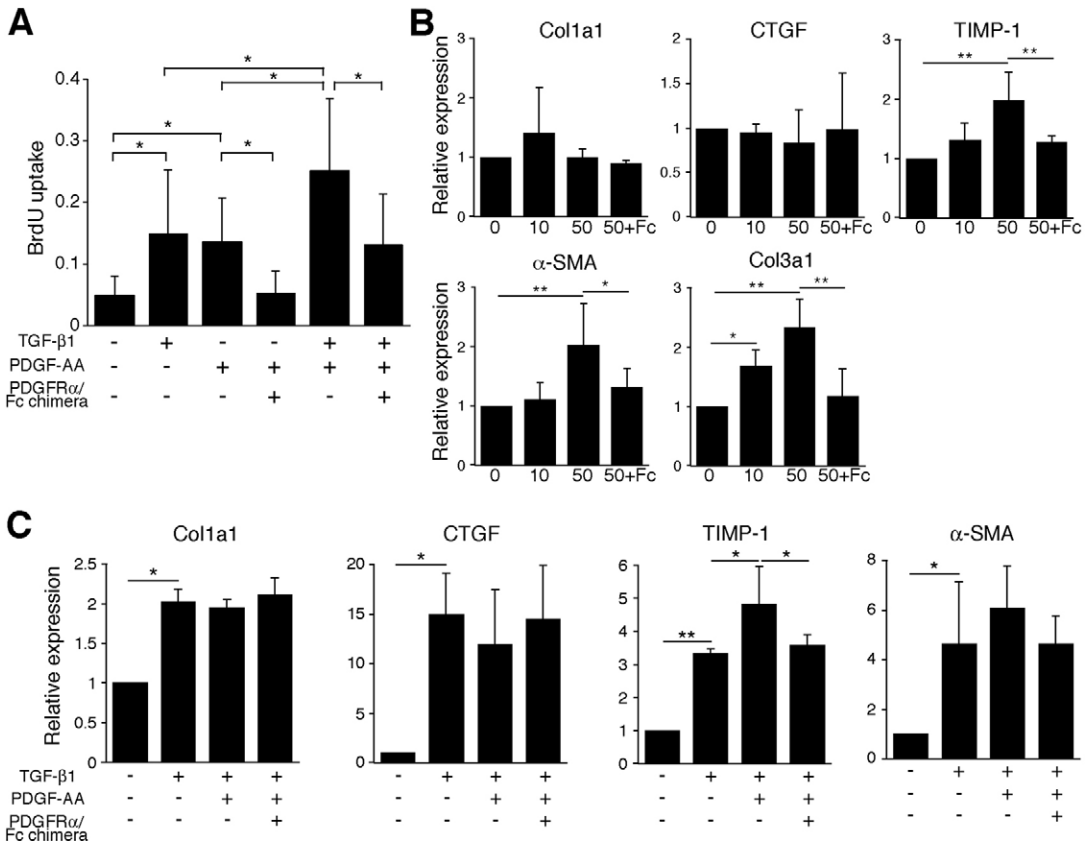

Fig. 8. PDGF-AA promotes proliferation and expression of fibrosis markers in PDGFRα$^+$ cells. (**A**) TGF-β1 and PDGF-AA additively promote proliferation of PDGFRα$^+$ cells. PDGFRα$^+$ cells isolated from regenerating muscle 3 days after cardiotoxin (CTX) injection were cultured with TGF-β1 (1 ng/ml), PDGF-AA ligand (10 ng/ml) and/or PDGFRα–Fc chimeric receptor (200 ng/ml) under serum-free conditions. Proliferation of PDGFRα$^+$ cells was analyzed by measuring the uptake of bromodeoxyuridine (5-bromo-2′-deoxyuridine) (n=6). *P<0.05: indicates experimental pairs where differences between the compared values were statistically significant. In this experiment, non-repeated measures analysis of variance (ANOVA) followed by the Student–Newman–Keuls (SNK) test were used. (**B**) PDGFRα$^+$ cells were incubated for 12 hours in serum-free DMEM, then exposed to 0–50 ng/ml PDGF-AA for 6 (Col1a1, CTGF, TIMP1 and α-SMA) or 48 (Col3a1) hours with or without PDGFRα–Fc chimeric receptor (200 ng/ml). Transcripts of fibrosis-associated molecules were quantified by qRT-PCR. *P<0.05, **P<0.01: indicate the level of significance of the differences. (**C**) PDGFRα$^+$ cells were cultured with or without TGF-β1 (1 ng/ml), PDGF-AA (50 ng/ml) and PDGFRα–Fc chimeric receptor (200 ng/ml), and the expression of mRNA encoding fibrosis markers was analyzed by qRT-PCR. *P<0.05, **P<0.01: indicate the level of significance of the differences.

mice (Zhou et al., 2006). Therefore, TGF-β isoforms and their signal pathways are a potent therapeutic target for DMD and other fibrotic diseases. Indeed, Andreetta et al. reported that an antibody neutralizing TGF-β reduced fibrosis in the *mdx* diaphragm, the most affected skeletal muscle in *mdx* mice (Andreetta et al., 2006). However, this treatment was accompanied by an increased inflammatory response. By contrast, Vidal et al. reported that fibrinogen induces TGF-β expression in macrophages through synthesis of IL-1β. Furthermore, genetic reduction or pharmacological depletion of fibrinogen led to the reduction of both fibrosis and inflammation in *mdx* mice (Vidal et al., 2008). Losartan, a blocker of the angiotensin II type I receptor, is also a potent therapeutic drug for DMD. Cohn et al. reported that losartan attenuates TGF-β-induced failure of skeletal muscle regeneration in *mdx* and fibrillin-null mice (Cohn et al., 2007). This treatment significantly suppressed fibrosis in the *mdx* diaphragm. This effect could be attributed to the reduced expression of thrombospondin-1 (TSP-1), which is a downstream target of angiotensin II and has the potential to activate latent TGF-β. Fibrosis-related molecules were upregulated in response to TGF-β in muscle mesenchymal progenitors (Fig. 4). In addition, they expressed mRNA encoding both TSP-1 and angiotensin II type I receptors in vitro (data not shown). Therefore, it could be considered that muscle mesenchymal progenitors are the target of these treatments.

Along with TGF-β, signaling between PDGFs and PDGFRs is also considered to be involved in several fibrotic diseases (Andrae et al., 2008). Although the importance of PDGF–PDGFRα signaling in muscular dystrophy is unclear, some studies have shown evidence of the importance of PDGFR signaling in skeletal muscle fibrosis. Zhao et al. observed strong protein expression of PDGF-A, PDGF-B, PDGFRα and PDGFRβ in human dystrophic muscle (Zhao et al., 2003). Recently, imatinib, an inhibitor of several tyrosine kinases, including c-abl, c-kit and PDGFRs, was demonstrated to ameliorate dystrophic phenotypes in *mdx* mice by suppressing the phosphorylation of PDGFRα (Huang et al., 2009). In addition, constitutively active PDGFRα-receptor knock-in mice exhibited systemic fibrosis, including that of skeletal muscle tissue (Olson and Soriano, 2009). In fact, PDGF-AA promoted the proliferation of PDGFRα$^+$ cells to the same degree as TGF-β (Fig. 8A).

Furthermore, PDGF-AA induced expression of Col3a1, α-SMA and TIMP1 (Fig. 8B,C). These results raise the possibility that PDGFRα is not only an excellent marker for the major contributor to muscle fibrosis but also a key functional molecule in the progression of muscle fibrosis.

The myogenic cell was also one of the candidates to produce ECM in fibrotic muscle. Alexakis et al. observed expression of collagen type I in myogenic cells by using primary myoblasts and C2C12, a myogenic cell line (Alexakis et al., 2007). Li et al. showed that overexpression of TGF-β1 stimulated C2C12 cells to differentiate into fibroblastic cells in vivo (Li et al., 2004). However, in this study, we showed that myogenic cells are much less sensitive to TGF-β isoforms than are PDGFRα$^+$ mesenchymal progenitors. Furthermore, because myogenic cells do not express PDGFRα, they cannot transduce the PDGFRα signal that should exert the stimulatory effect on muscle fibrosis described above. Thus, it seems that the contribution of myogenic cells to muscle fibrosis is very limited, whereas PDGFRα$^+$ cells are the major source of skeletal muscle fibrosis, at least under the dystrophic condition. Brack and colleagues also reported that myogenic cells become fibroblastic cells during aging in response to increased Wnt signaling (Brack et al., 2007). It remains to be seen which cell type – myogenic cells or PDGFRα$^+$ mesenchymal progenitors – is the main source of fibrogenic cells in an aging environment. However, given their highly fibrogenic characteristic, PDGFRα$^+$ mesenchymal progenitors should also be considered to be one of the best possible candidates for fibrosis-initiating cells in an aged environment.

Diverse features of muscle mesenchymal progenitors

In DMD patients, fat and collagen depositions are observed in skeletal muscle as dystrophic changes that progress with age. Our results suggest that fat accumulation and fibrosis originate from common PDGFRα$^+$ mesenchymal progenitors. However, it is unclear how mesenchymal progenitors are regulated to differentiate into either adipocytes or collagen-producing cells. In addition, Joe and colleagues have reported that CD31$^-$ CD45$^-$ Inga7$^-$ Sca-1$^+$ cells (identical to PDGFRα$^+$ mesenchymal progenitors) promote normal skeletal muscle regeneration (Joe et al., 2010; Rodeheffer, 2010). Therefore, these mesenchymal progenitors have two distinct roles: one is to promote skeletal muscle regeneration, whereas the other is to contribute to pathogenic changes. A most important task is to elucidate the diverse differentiation mechanisms of muscle mesenchymal progenitors. Targeting PDGFRα$^+$ mesenchymal progenitors might open new opportunities for designing therapeutic strategies for muscle diseases.

Materials and Methods

Mice

Eight-week-old specific pathogen-free C57BL/6 mice were purchased from Charles River Japan (Yokohama, Kanagawa, Japan). Specific pathogen-free *mdx* mice (of C57BL/10 background) were provided by Central Laboratories of Experimental Animals (Kawasaki, Kanagawa, Japan) and maintained in our animal facility by brother–sister matings. Heterozygous GFP-tg mice (Okabe et al., 1997) with a C57BL/6 background were maintained in our animal facility by mating with normal C57BL/6 mice. All procedures for experimental animals were approved by the Experimental Animal Care and Use Committee at Osaka University.

Muscle injury

Muscle injury was induced by injecting cardiotoxin (10 μM in saline, Sigma-Aldrich, St Louis, MO) into tibialis anterior (50 μl), gastrocnemius (150 μl) and quadriceps femoris (100 μl) muscles. All injections were performed when mice were 8–10 weeks of age.

Preparation of mononuclear cells from skeletal muscle

Mononuclear cells from diaphragms of *mdx* mice, regenerating limb muscles 3 days after cardiotoxin injection or uninjured muscles were prepared using 0.2% collagenase type II (Worthington Biochemical Corp., Lakewood, NJ). Mononuclear cells were stained with FITC-conjugated anti-CD31 (BD Pharmingen, San Diego, CA), FITC-conjugated anti-CD45 (BD Pharmingen) and biotinylated anti-PDGFRα (R&D Systems, Minneapolis, MN) antibodies. Cells were then incubated with streptavidin-labeled allophycocyanin (BD Biosciences, San Diego, CA) or streptavidin-labeled phycoerythrin (Dako North America, Carpinteria, CA) on ice for 30 minutes and resuspended in PBS containing 2% FBS and 2 mg/ml propidium iodide (PI). Cell sorting was performed using an FACS VantageSE or Aria II flow cytometer (BD Immunocytometry Systems, Mountain View, CA). Debris and dead cells were excluded by forward scatter, side scatter and PI gating. Data were collected using CELLQuest or FACSDiva software (BD Biosciences). An IMag Immunocytometry system (BD Immunocytometry Systems) was also used for isolation of PDGFRα$^+$ and PDGFRα$^-$ cells from injured or uninjured muscles.

Isolation and culture of muscle satellite cells

Satellite cells were isolated from intact adult skeletal muscle using biotinylated-SM/C-2.6 (Fukada et al., 2004) and IMag methods, as described previously (Fukada et al., 2008). Satellite cells were cultured in a growth medium of high-glucose DMEM (Sigma-Aldrich) containing 20% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS), 10 ng/ml recombinant human bFGF (PeproTech, London, UK), 25 ng/ml recombinant mouse HGF (R&D Systems), 5 μg/ml heparin (Wako Pure Chemical Industries, Osaka, Japan) and penicillin (100 U/ml)-streptomycin (100 μg/ml) (Gibco-BRL, Gaithersburg, MD) on culture dishes coated with Matrigel (BD Biosciences).

Induction of fibrotic and adipogenic cells

Freshly isolated cells were cultured for 3 days in high-glucose DMEM glucose (Sigma-Aldrich) containing 10% FCS and penicillin–streptomycin on eight-well Lab-Tek Chamber slides (Nalge Nunc International, Rochester, NY). For fibrotic differentiation, the cells were additionally cultured with TGFβ1–TGFβ3 for 3–5 days (Sigma-Aldrich). For adipogenic differentiation, the cells were additionally cultured in adipogenic induction medium (Cambrex Bioscience, Walkersville, MD) for 3 days and then cultured for 1 day in adipogenic maintenance medium (Cambrex Bioscience). This procedure was repeated three times. Then cells were maintained for 5 more days in the adipogenic maintenance medium as described previously (Uezumi et al., 2006). The adipogenic induction medium contained DMEM supplemented with 10% FCS and antibiotics plus 0.2 mM indomethacin, 0.5 mM 3-isobutyl-1-methyl-xanthine, 10 μg/ml recombinant human insulin and 1 μM dexamethasone. The adipogenic maintenance medium contained DMEM supplemented with 10% FCS and antibiotics plus 10 μg/ml recombinant human insulin.

To stain lipids, cells were fixed in 10% formalin, rinsed in water and then in 60% isopropanol, stained with Oil red O in 60% isopropanol and rinsed in water again.

Clonal culture and analyses

Clones derived from a single PDGFRα$^+$ cell were obtained from sorted cells using a Cellmatrix Collagen cell culturing kit according to the manufacturer's instruction (Nitta Gelatin, Osaka, Japan). Seven volumes of Cellmatrix Type I-A, two volumes of 5× concentration DMEM-high glucose and one volume of reconstitution buffer were mixed to prepare a collagen base layer medium. It was then put into a six-well culture dish and incubated at 37℃ for 30 minutes. A cell layer including 100–200 PDGFRα$^+$ cells in the collagen base layer medium was placed on top of the base layer and incubated at 37℃ for 30 minutes. The cell layer was overlaid with high-glucose DMEM glucose (Sigma-Aldrich) containing 20% FCS (JRH Biosciences), 10 ng/ml recombinant human bFGF and penicillin–streptomycin. Cells were cultured for 4–8 weeks, with an upper medium replacement every 2 days. Then the culture medium was removed, and clones (including approximately 2×10^4–1×10^5 cells) were picked up and digested with 0.2% collagenase type II (Worthington Biochemical) to obtain the cell suspension. The cells were additionally cultured in fibrotic or adipogenic differentiation medium.

RT-PCR

Real-time PCR analysis was performed as described previously (Segawa et al., 2008). Briefly, total RNA was extracted from cultured or sorted cells with a Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and then reverse-transcribed into cDNA using TaqMan Reverse Transcription Reagents (Roche Diagnostics, Mannheim, Germany). Total RNA of diaphragms derived from 9-week-old C57BL/6 and

13-week-old *mdx* mice was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's manual.

Real time PCR was performed using SYBR Premix Ex Taq (Takara, Kyoto, Japan) at a final volume of 10 µl. All primer combinations were positioned to span an intron between two exons. Specific forward and reverse primers to produce approximately 100-bp amplicons for optimal amplification in real-time PCR of reverse-transcribed cDNAs were 5′-TCATCGTGGCTTCTCTGGTC and 5′-GACCGTTGAGTCCGTCTTTG for mouse Col1a1; 5′-ACGTAAGCACTGGTGGACAGA and 5′-GAGGGCCATAGCTGAACTGA for mouse Col3a1; 5′-TCCACCCGAGTTACCAA and 5′- TTAGGTGTCCGGATGC for mouse CTGF; 5′-ACGCTGAAGTATCCGA and 5′-CATTTTCTCCCGGTTGG for mouse α-SMA; 5′-AGCCTCTGTGGATATGCCC and 5′-TCAGAGTACGCCAGGGAACC for mouse TIMP1; 5′-GCCTGAGTGGCTGTCTTTTGA and 5′-CACAAGAGCAGTGAGCGCTGAA for mouse TGF-β1; 5′-CGAGGCGAGATTTGCAGGTATT and 5′-TTAGCAGGAGATGTGGGGTCTT for mouse TGF-β2; 5′-AGATACTTCGACCGGATG and 5′-GAGTCCAACTTGGGTCT for mouse TGF-β3; and 5′-CTTTGCTGACCTGCTGGATTACAT and 5′-GTCCCCCGTTGACTGATCATTAC for mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT). Real-time PCR and data analyses were performed on a LightCycler quick-system 350S using LightCycler Software (Roche Diagnostics). Samples were amplified, and the relative gene expression levels were calculated using standard curves generated by serial dilutions of the cDNA. The primers, which produced approximately 500-bp amplicons using the standard curves, were 5′-TGACAAGGGTGAGACAGG C and 5′-ACCACGTTAGCATCATCGGC-3′ for mouse Col1a1; 5′-GACCTAAAATTCTGCCACCCCG-3′ and 5′-TGGTATGTAATGTTCTGGGAGGC-3′ for mouse Col3a1; 5′-TCCACCCGAGTTACCAA-3′ and 5′-GGATAGTTCCTCCCACG-3′ for mouse CTGF; 5′-CCATCCATCGTGGGAC-3′ and 5′-GGACAATCTCACGCTCG-3′ for mouse α-SMA; 5′-AAAGGATTCAAGGCTGTGGG-3′ and 5′-GCAGGCAAGCAAAGTGACG-3′ for mouse TIMP1; 5′-CTAATGGTGGACCGCAACAAC-3′ and 5′-CGGTTCATGTCATGGATGGTG-3′ for mouse TGF-β1; 5′-AAAATGCCATCCCGCCCACTT-3′ and 5′-TTAGCAGGAGATGTGGGGTCTT-3′ for mouse TGF-β2; 5′-TGGCGGAGCACAATGAACTG-3′ and 5′-GGATAGTTCCTCCCACG-3′ for mouse TGF-β3; and 5′-CTTTGCTGACCTGCTGGATTACAT-3′ and 5′-GTCAAGGGCATATCCAACAACAAA-3′ for mouse HPRT.

Specific primer sequences for conventional RT-PCR were 5′-AAGTTCGGGAAGAAAGAGGACGAC and 5′-GAGGTCGGGTTCTGATTCCACATC for mouse Pax7; 5′-GTCAACCAAGCTTTCGAGACG-3′ and 5′-CGGAGCTTTTATCTGCAGCAC-3′ for mouse Myf5; 5′-TCTGTGGTTCTGCGTGGAGA-3′ and 5′-GTATCATTTCCAACCACCCT-3′ for mouse Flk1; 5′-GCGGCAATAGGACAAACAC-3′ and 5′-GATGAAGATGGGAGCGATG-3′ for mouse vWF; 5′-GACGAGTGTCCTTCGCCAAAGTG-3′ and 5′-CAAAATCCGACCAAGCACGAGG-3′ for mouse PDGFRα; 5′-AGGTCCAGGGAGGTTGTGA-3′ and 5′-CCGCCATGTAGTCCAGGTAG-3′ for mouse CD68; 5′-AAGCATCCGAGACACACACA-3′ and 5′-GGCAAGACATACCAGGGAGA-3′ for mouse F4/80; 5′-GCAACCCTGGAACAAATGG-3′ and 5′-GCTCACCAACAAGTCCTCTGG-3′ for mouse Col1a2; 5′-GAAAGGTGAGCCAGGAGACA-3′ and 5′-CCCAGCCAGACCATCAAAG-3′ for mouse Col5a1; 5′-TGAAGGAACAGCAGGAAATGA-3′ and 5′-CACCACGAGAACCAGGATTG-3′ for mouse Col5a2; 5′-ACTCCCACCCACACAGAACA-3′ and 5′-GCCACAAAGCCAAACACATC-3′ for mouse Col6a1; 5′-CCATCAACCGCATCATCAA-3′ and 5′-TCCCTGTCGTCCTTTCTGTC-3′ for moue Col6a2; 5′-ACAATGCCGTGGTCCTAACA-3′ and 5′-AGTGAATGAGTTGGCGGTGA-3′ for mouse Fn1; 5′-TGCCACCTCGTGTGAAGAA-3′ and 5′-TGCTGGCTGGACTCTCAATC-3′ for mouse Itgb5; 5′-CGTCGCTGGGATGATGTAG-3′ and 5′-GGGTCTGGGATAGGAATAGCA-3′ for mouse Lamb2; 5′-TGAAGACGGAGTGGGAGATG-3′ and 5′-TTGTCC TTGGTGTCCTGGTG-3′ for mouse Thbs3.

Skeletal muscle fixation and immunohistological analysis (H&E, Sirius red and Masson trichrome staining)

Isolated muscles were fixed in 4% paraformaldehyde (PFA) in PBS for 30 minutes, and muscles were sequentially soaked in 10% sucrose in PBS and 20% sucrose in PBS. Muscles were then frozen in liquid-nitrogen-cooled isopentane (Wako Pure Chemical Industries). Cryosections (10 µm) were stained with hematoxylin and eosin (H&E), Sirius red (Sigma-Aldrich) or Masson trichrome (Muto Pure Chemicals, Tokyo, Japan).

Immunohistological studies (fluorescent staining)

For immunohistochemistry examinations, transverse cryosections (6 µm) were stained with anti-laminin α2 (Alexis Biochemicals, San Diego, CA), anti-collagen type I (Biogenesis, Poole, UK) and anti-GFP (Chemicon International, Temecula, CA) antibodies. After the first staining at 4℃ overnight, sections were incubated with secondary antibodies conjugated to Alexa Fluor 568 or Alexa Fluor 488 (Molecular Probes, Eugene, OR). Coverslips were mounted using Vectashield (Vector Laboratories, Burlingame, CA). The signals were recorded photographically using a fluorescence microscope BX51 (Olympus, Tokyo, Japan).

Immunocytochemistry

Freshly isolated PDGFRα$^+$ cells were cultured with TGF-β1 in eight-well Lab-Tek Chamber slides (Nalge Nunc International) for 3 days. Cells were then fixed using 4% PFA and permeabilized by 0.25% Triton X-100. Cells were stained with antibodies against collagen type I (Biogenesis) or α-SMA (Sigma-Aldrich) at 4℃ overnight, then were reacted with secondary antibodies conjugated to Alexa Fluor 568 or Alexa Fluor 488 (Molecular Probes). Nuclei were stained with DAPI. The signals were recorded photographically using a fluorescence microscope BX51 (Olympus).

Cell transplantation

C57BL/6 tibialis anterior (TA) muscles were injected with cardiotoxin 1 or 3 days before gamma-ray irradiation (8 Gy) using a Gamma Cell ^{137}Cs source (MDS Nordion, Ottawa, Ontario, Canada). Then, 5×10^5 PDGFRα$^-$, 1.5×10^5 PDGFRα$^+$ or 1×10^5 primary myoblasts were injected into the TA muscle within 3 hours after irradiation. C57BL/6-derived bone marrow cells were also transplanted into recipient mice that had been treated with ampicillin (Sigma-Aldrich). Ampicillin was put in the drinking water (1 g/l) for 2 weeks after irradiation and transplantation. Two weeks after transplantation, the TA muscles were fixed in 2% PFA.

Bone marrow chimera

Adult *mdx* mice were irradiated with gamma rays (8 Gy) using a Gamma Cell ^{137}Cs source (MDS Nordion). Within 3 hours after irradiation, 1×10^7 bone marrow cells from GFP-tg mice were injected intravenously into recipient mice. About 9–10 months after transplantation, diaphragms were isolated and then fixed in 2% PFA.

Microarray analysis

PDGFRα$^+$ cells and myogenic cells from *mdx* diaphragm were sorted. In total, 10 *mdx* mice (23–26 weeks old) were used to collect the cells. RNA was purified using an RNeasy Micro Kit (Qiagen). Microarray analysis was performed using a 3D-Gene Mouse Oligo chip 24 k (Toray, Tokyo, Japan). Total RNA was labeled with Cy3 or Cy5 using the Amino Allyl MessageAMP II aRNA Amplification Kit (Life Technologies, Carlsbad, CA). The hybridization was performed using the supplier's protocols (www.3d-gene.com). Hybridization signals were scanned using ScanArray Express Scanner (PerkinElmer, Waltham, MA) and processed by GenePixPro version 5.0 (Molecular Devices, Sunnyvale, CA). Detected signals for each gene were normalized by the global normalization method (Cy3:Cy5 ratio median=1). The microarray data were deposited at Gene Expression Omnibus under accession number GSE25258.

Cell proliferation assay

PDGFRα$^+$ cells derived from regenerating muscles were seeded in 96-well culture plates (Asahi Glass, Tokyo, Japan) and were cultured in DMEM containing 10% FCS and antibiotics. After 3 days, the PDGFRα$^+$ cells were incubated for 12 hours in serum-free DMEM containing 1 ng/ml TGF-β1, 10 ng/ml recombinant human PDGF-AA (R&D Systems) and/or 200 ng/ml recombinant mouse PDGFRα–Fc chimeric receptor (R&D Systems). At the same time, 10 µM BrdU (5-bromo-2′-deoxyuridine) was added in each well. Then, the cells were fixed, and nuclear incorporation of BrdU was measured by a cell proliferation enzyme-linked immunosorbent assay (Roche Diagnostics).

Statistics

Values were expressed as means ± s.d. Statistical significance was assessed by a Student's *t*-test. To compare more than two groups, non-repeated measures analysis of variance (ANOVA) followed by a Bonferroni test were used. A probability of less than 5% ($P<0.05$) or 1% ($P<0.01$) was considered statistically significant.

Acknowledgements

We thank K. Takeda (Osaka University) for use of a Gamma Cell ^{137}Cs source. We also thank Katherine Ono for reading this manuscript.

Funding

This work was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI [grant numbers 18890216 to A.U., 18800023 to S.F.]; The Ministry of Education, Culture, Sports, Science and Technology (MEXT) KAKENHI [grant numbers 21790884 to A.U., 16300132 to H.Y.]; a research grant for study of nervous and mental disorders from the Ministry of Health, Labour and Welfare [19A-7 to S.T. and H.Y.]; Intramural Research Grant for Neurological and Psychiatric Disorders of The National Center of

Neurology and Psychiatry [grant number 22-1 to S.F.]; and the Osaka Foundation for Promotion of Clinical Immunology (to S.F.).

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.086629/-/DC1

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