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ORIGINAL ARTICLE

Neer Award 2018: Platelet-derived growth factor receptor α co-expression typifies a subset of platelet-derived growth factor receptor β–positive progenitor cells that contribute to fatty degeneration and fibrosis of the murine rotator cuff

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Background and hypothesis: After massive tears, rotator cuff muscle often undergoes atrophy, fibrosis, and fatty degeneration. These changes can lead to high surgical failure rates and poor patient outcomes. The identity of the progenitor cells involved in these processes has not been fully elucidated. Platelet-derived growth factor receptor β (PDGFRβ) and platelet-derived growth factor receptor α (PDGFRα) have previously been recognized as markers of cells involved in muscle fibroadipogenesis. We hypothesized that PDGFRα expression identifies a fibroadipogenic subset of PDGFRβ$^+$ progenitor cells that contribute to fibroadipogenesis of the rotator cuff.

Methods: We created massive rotator cuff tears in a transgenic strain of mice that allows PDGFRβ$^+$ cells to be tracked via green fluorescent protein (GFP) fluorescence. We then harvested rotator cuff muscle tissues at multiple time points postoperatively and analyzed them for the presence and localization of GFP$^+$ PDGFRβ$^+$ PDGFRα$^+$ cells. We cultured, induced, and treated these cells with the molecular inhibitor CWHM-12 to assess fibrosis inhibition.

This study was presented at the 80th Annual Meeting of the Western Orthopaedic Association; September 29, 2016; Indian Wells, CA, USA.
This study received ethical approval from the University of California, Los Angeles Institutional Animal Care and Use Committee (ARC No. 2012-042-11C).

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Results: GFP$^+$ PDGFRβ$^+$ PDGFRα$^+$ cells were present in rotator cuff muscle tissue and, after massive tears, localized to fibrotic and adipogenic tissues. The frequency of PDGFRβ$^+$ PDGFRα$^+$ cells increased at 5 days after massive cuff tears and decreased to basal levels within 2 weeks. PDGFRβ$^+$ PDGFRα$^+$ cells were highly adipogenic and significantly more fibrogenic than PDGFRβ$^+$ PDGFRα$^-$ cells in vitro and localized to adipogenic and fibrotic tissues in vivo. Treatment with CWHM-12 significantly decreased fibrogenesis from PDGFRβ$^+$ PDGFRα$^+$ cells.
Conclusion: PDGFRβ$^+$ PDGFRα$^+$ cells directly contribute to fibrosis and fatty degeneration after massive rotator cuff tears in the mouse model. In addition, CWHM-12 treatment inhibits fibrogenesis from PDGFRβ$^+$ PDGFRα$^+$ cells in vitro. Clinically, perioperative PDGFRβ$^+$ PDGFRα$^+$ cell inhibition may limit rotator cuff tissue degeneration and, ultimately, improve surgical outcomes for massive rotator cuff tears.
Level of evidence: Basic Science Study; Molecular and Cell Biology; Animal Model

Keywords: Rotator cuff; massive rotator cuff tear; rotator cuff repair; fatty degeneration; fibroadipogenic progenitor cell; PDGFRα; PDGFRβ

Rotator cuff tears affect an estimated 10% of the population over the age of 60 years, leading to significant activity-related pain and decreased quality of life.[11] Over 75,000 rotator cuff repair surgical procedures are performed annually in the United States alone.[45]

While the outcomes of rotator cuff repair surgical procedures are generally positive, surgical failures lead to significant morbidity.[18] Failures often require costly revision surgery or salvage procedures such as reverse total shoulder arthroplasty.[38] Tendon retears are the leading cause of surgical failure, and the rate of retear or persistent tear is strongly associated with muscle atrophy and fatty degeneration.[7] Massive rotator cuff tears are associated with significant fatty degeneration and the highest rates of tendon retear, with reported incidences of up to 94%.[36]

After rotator cuff tears, the severity of fibroadipogenesis, which is the pathologic process characterized by fatty degeneration and fibrotic scar formation, correlates with increasing tear size, patient age, and duration of the tear.[34] Fatty degeneration in particular has been associated with poor surgical outcomes and is the basis of the Goutallier rotator cuff tear staging system.[5,24] These degenerative processes lead to decreased muscle strength and tissue compliance, making surgical repair and biological healing challenging.[34]

The cellular origin of these fibroadipogenic degenerative changes noted in massive rotator cuff tears remains unclear. Researchers have identified a candidate mesenchymal stem cell population in skeletal muscle that possesses both fibrotic and adipogenic potential on differentiation.[22,25,42] A number of different antigen combinations—including CD31$^-$, CD45$^-$, integrin α7$^-$, and stem cells antigen-1$^+$[22]; CD31$^-$, CD45$^-$, and platelet-derived growth factor receptor α (PDGFRα) positive[43]; and CD31$^-$, CD45$^-$, integrin α7$^-$, stem cells antigen-1$^+$, and PDGFRα$^+$—have characterized these progenitor cells, which are sometimes referred to as "fibroadipogenic progenitor (FAP) cells."[25] Researchers have identified PDGFRα, in particular, as a reliable marker of FAP cells,[19] while platelet-derived growth factor receptor β (PDGFRβ)–positive cells have been identified in muscle tissue as a marker of some progenitor cells that are implicated in pathologic tissue fibrosis.[15] Researchers have found that PDGFRα$^+$ cells are involved in fibrotic and adipogenic muscle degeneration following toxin-mediated injury to the hindlimb or injury to the rotator cuff of animals with muscular dystrophy, as well as in age-associated sarcopenia.[1,19,30,41,43] Dulauroy et al[9] demonstrated that a subset of PDGFRα$^+$ cells expressing a disintegrin and metalloproteinase protein–12 possess increased fibrogenic potential and that ablation of these cells decreases pathologic fibrosis.

Through targeting the α_v subunit of integrins in fibrogenic myofibroblasts by the administration of CWHM-12, a small α_v integrin inhibitor, researchers have effectively reduced transforming growth factor β1 (TGF-β1)–mediated fibrosis in the liver, kidney, and lung.[17] Researchers have identified PDGFRα$^+$ cells in human skeletal muscle tissue as well, although the role of these cells in human pathology, including rotator cuff muscle degeneration, remains largely undefined.[41]

To establish whether co-expression of PDGFRα and PDGFRβ identifies a population of profibrotic and proadipogenic cells involved in muscle tissue degeneration following massive rotator cuff tears, we examined the capacity for purified populations of these cells to differentiate into adipogenic and fibrotic cell lineages in culture. Furthermore, we used this validated model of massive rotator cuff tears in a transgenic mouse to evaluate the contribution of these cells to fibrosis and fatty degeneration in vivo. Last, to demonstrate the potential for inhibiting degradative tissue processes from PDGFRβ$^+$ PDGFRα$^+$ cells, we examined the ability of CWHM-12 to inhibit fibrogenesis after massive rotator cuff tears in vitro. We show that rotator cuff–derived PDGFRβ$^+$ PDGFRα$^+$ cells have fibrogenic and adipogenic potentials in vitro, directly contribute to fibroadipogenesis after massive rotator cuff tears in vivo, and can be inhibited through treatment with CWHM-12 in vitro.

Materials and methods

This is a laboratory study using transgenic mice to evaluate the etiology of rotator cuff fibrosis and fatty degeneration following massive rotator cuff tears.

Pdgfrb-BAC-eGFP mice

We obtained mTmG (dTomato-EGFP)[21] mice from The Jackson Laboratory (Bar Harbor, ME, USA) and crossed them with *Pdgfrb*-CRE mice. *Pdgfrb*-BAC-eGFP reporter mice (on a C57BL/6 background) carry 1 copy of a bacterial artificial chromosome (BAC) transgene expressing enhanced green fluorescent protein (eGFP) under the control of the *Pdgfrb* regulatory elements. They originate from the Gene Expression Nervous System Atlas project, and we obtained them from the Mutant Mouse Resource and Research Center [STOCK Tg(Pdgfrb-EGFP)JN169Gsat/Mmucd]. We performed genotyping of all mice via polymerase chain reaction (PCR). We randomly divided the *Pdgfrb*-BAC-eGFP reporter mice, at 8-10 weeks old, into 1 of 3 groups: healthy uninjured group (3 mice), sham-surgery group (12 mice), or supraspinatus tendon tenotomy and suprascapular neurotomy (tenotomy and denervation [TT-DN]) group (12 mice).

Surgical procedures

We performed the massive rotator cuff tear surgical procedure as previously described.[29] In brief, we anesthetized the mice with 2% isoflurane and oxygen, administered buprenorphine for analgesia, and sterilely prepared and draped the right shoulder. For the TT-DN group, we made a 1-cm skin incision longitudinally over the right glenohumeral joint. We then split the deltoid fibers directly posterior to the deltoid tuberosity longitudinally and identified the supraspinatus and infraspinatus tendons. We isolated and sharply dissected the supraspinatus and infraspinatus tendons from their insertions on the greater tuberosity with a No. 11 blade scalpel for the tenotomy procedure and resected the distal 5 mm of each tendon to prevent scar formation to the humerus. Next, we identified the suprascapular nerve through a 5-mm incision in the trapezius musculature anterior to the lateral scapula and cut this nerve for the denervation procedure. Last, we closed the deltoid muscle and skin with No. 5-0 Vicryl (Ethicon, Somerville, NJ, USA).

For the sham procedure, we performed the same skin incision and muscle dissections, but the tendon transection and denervation procedures were not performed. We euthanized the mice at 5 days, 2 weeks, 4 weeks, or 6 weeks for subsequent analyses. The local institutional animal care and use committee approved all animal procedures.

Skeletal muscle dissociation

We excised the supraspinatus and infraspinatus muscles following TT-DN, following sham surgery, or in healthy uninjured mice at the indicated time points; mechanically minced the tissue; and dissociated them using 0.5-mg/mL Collagenase II and Dispase (Sigma, St Louis, MO, USA) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep) for 30 minutes at 37°C on a shaker. We then filtered cell suspensions through 100- and 0.45-μm strainers (BD Biosciences, San Jose, CA, USA), washed them in phosphate-buffered saline solution (PBS), and centrifuged them for 10 minutes at 335*g*. With the freshly isolated cells, we either analyzed them by flow cytometry or further cultured them in DMEM supplemented with 20% FBS and 1% Pen-Strep.

Flow cytometry and cell sorting

We washed freshly isolated cells—or cultured cells from the rotator cuff musculature—in PBS, centrifuged them, and labeled them with PE/Cy7-conjugated anti-mouse PDGFRα and APC-conjugated anti-mouse PDGFRβ (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. We performed fluorescence-activated cell sorting (FACS) using the Aria II flow cytometer (BD Biosciences), excluding debris and dead cells according to forward and side scatter, and we used the LSR II flow cytometer and BD FACSDiva (BD Biosciences) or FlowJo software (FlowJo, Ashland, OR, USA) for subsequent analyses.

Histology and immunohistochemistry

We fixed sections from the rotator cuff musculature in 4% formalin; embedded them in paraffin; and then sectioned, dehydrated, and stained them with hematoxylin-eosin for general tissue structure or Picro-Sirius Red (PSr; Abcam, Cambridge, UK) for collagen expression, according to the manufacturer's instructions. We acquired images with the Axio Imager 2 light microscope (Zeiss, Oberkochen, Germany) and quantified PSr-stained areas for collagen density quantification by selecting red pixels on Photoshop CS6 Extended (Adobe, San Jose, CA, USA) and calculating the percentage out of total pixels from randomly distributed tissue sections.

For fluorescence microscopy, we fixed frozen sections with 4% paraformaldehyde, washed them 3 times in PBS, immunolabeled them with rabbit anti-mouse PDGFRβ and goat anti-mouse PDGFRα overnight at 4°C, washed them 3 more times in PBS, and then incubated them with Alexa Fluor 647–conjugated donkey anti-rabbit and Alexa Fluor 405–conjugated donkey anti-goat secondary antibodies (Abcam). We acquired images with the SP5 confocal microscope (Leica, Wetzlar, Germany).

In vitro differentiation assays

We cultured freshly isolated whole rotator cuff cells for 3-5 days and then further sorted them based on the expression of green fluorescent protein (GFP), PDGFRα, and PDGFRβ with FACS. We cultured these cells for up to 2 passages and exposed them to adipogenic or fibrotic differentiation media. For fibrotic differentiation, we cultured the cells in DMEM with 10% FBS and 1% Pen-Strep with 1-ng/mL mouse TGF-β1 (R&D Systems, Minneapolis, MN, USA) for 10-14 days, changing the media every 3-5 days. We identified collagen production from the induced cells with PSr stain according to the manufacturer's instructions (Abcam). For collagen quantification, we incubated the cells in 0.1-mol/L sodium hydroxide for 1 hour at room temperature after PSr staining. We removed 200 μL of the resulting supernatant and then measured each sample's absorbance at 560 nm on the Infinite 200 PRO spectrophotometer (Tecan, Männedorf, Switzerland) and compared this value

with a standard curve. For adipogenic differentiation, we cultured cells in DMEM supplemented with 10% FBS, 1-µmol/L dexamethasone, 1-µmol/L insulin, 0.5-mmol/L 3-isobutyl-1-methylxanthine, and 1% Pen-Strep for 5 days. We assessed adipogenic differentiation with Oil Red O staining (ScienCell Research Laboratories, San Diego, CA, USA) for the presence of lipids according to the manufacturer's instructions.

For in vitro fibrosis inhibition experiments, we cultured PDGFRβ$^+$ PDGFRα$^+$ cells in the presence of 1-ng/mL TGF-β1 with or without 10-nmol/L CWHM-12, an α_v integrin inhibitor (AOBIOUS, Gloucester, MA, USA).[44] Non-induced cultured PDGFRβ$^+$ PDGFRα$^+$ cells served as matched controls.

RNA extraction and reverse transcription PCR

Immediately following harvest, we froze the infraspinatus and supraspinatus muscle tissue from each animal in liquid nitrogen and stored it at −80°C. We isolated RNA from muscle tissue using TRI Reagent Solution (Molecular Research Center, Cincinnati, OH, USA) and measured RNA concentrations with NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). We then reverse transcribed RNA to complementary DNA using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA, USA) and the iCycler thermal cycler (BioRad). We ran the PCRs using 130-200 ng of RNA under the following cycling conditions: 5 minutes at 25°C for priming, followed by 20 minutes at 46°C for reverse transcription, and finally, 1 minute at 95°C for reverse transcriptase inactivation. We quantified the complementary DNA using Absolute SYBR Green Low ROX qPCR Mix (Life Technologies, Carlsbad, CA, USA) and the ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following cycling conditions: 15 minutes at 95°C for enzyme activation, followed by 40 cycles of amplification (15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C). We then analyzed the reverse transcription quantitative PCR data by calculating the fold change (2-ΔΔCt). Primer sequences (Integrated DNA Technologies, Coralville, IA, USA) are listed in Table I.

Statistical analysis

We used single-factor analysis of variance and the 2-tailed Student *t* test to compare mean values among study groups and the Tukey HSD (honest significant difference) test (equal sample sizes) and Tukey-Kramer test (unequal sample sizes) for post hoc analyses (Excel, version 14.5.7; Microsoft, Redmond, WA, USA). For all analyses, we considered $P < .05$ statistically significant.

Results

Murine rotator cuff muscle tissue undergoes predictable fibrosis and fatty degeneration in vivo following massive tendon tears

To confirm that murine rotator cuff tissue underwent fibrosis and fatty degeneration following the TT-DN procedure, we compared surgical mice with both the healthy uninjured mice and the sham-surgery mice at multiple time points over a period of 6 weeks. Histologic examination with hematoxylin-eosin staining revealed that the supraspinatus and infraspinatus muscles underwent significant and progressive muscle atrophy following TT-DN over the course of 6 weeks (Fig. 1, *A-D*). Compared with healthy uninjured rotator cuff muscle tissue (Fig. 1, *A*), in which few nuclei were present and they were consistently located at the myofiber periphery, we detected a rapid and robust accumulation of cells in the interstitial spaces between myofibers in supraspinatus and infraspinatus tissue at 5 days after TT-DN (Fig. 1, *B*). At 2 weeks after TT-DN, rotator cuff cellularity had decreased relative to the 5-day time point (Fig. 1, *C*) but was accompanied by increased adipocyte infiltration (Fig. 1, *C*, *G*, and *H*) and accumulation of Oil Red O–positive lipids in myofibers (Fig. 1, *L*) relative to both control (Fig. 1, *E* and *I*) and 5 days after TT-DN tissue (Fig. 1, *F* and *J*). We detected the development of PSr-positive fibrotic tissue at 2 weeks after TT-DN (Fig. 1, *C* and *P*), which was most severe at 6 weeks after TT-DN (Fig. 1, *Q*). At 6 weeks after TT-DN, supraspinatus and infraspinatus muscle tissues were severely atrophied with massive fatty degeneration, particularly at the infraspinatus, and were noted to have a significant accumulation of lipids within myofibers (Fig. 1, *H* and *M*) in addition to the increased collagen deposition (Fig. 1, *Q*). Altogether, these findings demonstrate the clinical relevance and reliability of the TT-DN model of massive rotator cuff tears and imply the presence of adipogenic and fibrotic progenitor cells residing within the skeletal muscle.

PDGFRβ$^+$ progenitor cells from rotator cuff muscle cause fibrosis and adipogenesis in vitro in time-dependent manner following massive tendon tears

To determine the role of PDGFRβ$^+$ cells in fibroadipogenic degeneration of muscle tissue following massive rotator cuff

Table I Murine primer sequences (5'-3')

	Forward primer	Reverse primer	Product length
GADPH	CCTGGAGAAACCTGCCAAGTATG	AGAGTGGGAGTTGCTGTTGAAGTC	133 bases
Leptin	TCCTGTGGCTTTGGTCCTATC	ATACCGACTGCGTGTGTGAA	129 bases
Col3A1	AGGCTGAAGGAAACAGCAAA	TAGTCTCATTGCCTTGCGTG	116 bases

GADPH, glyceraldehyde 3-phosphate dehydrogenase; *Col3A1*, collagen type III alpha 1 chain.

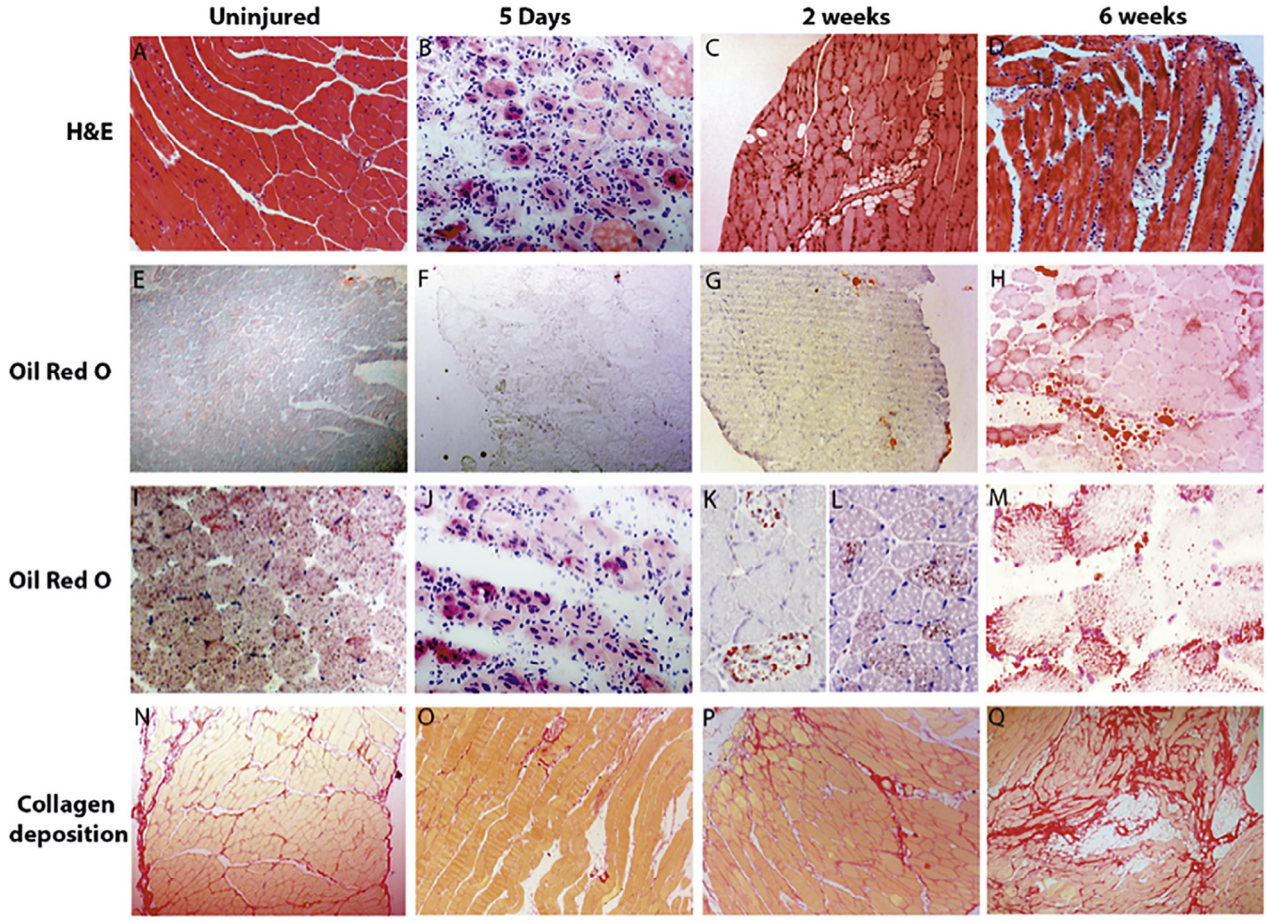

Figure 1 Representative images of progressive tissue fibrosis and fatty degeneration following massive rotator cuff tear in mouse. Healthy uninjured supraspinatus muscle tissue demonstrated normal muscle architecture (original magnification ×50) (**A**), minimal fatty degeneration (original magnification ×50 and ×400) (**E, I**), and minimal fibrosis (original magnification ×100) (**N**). At 5 days, 2 weeks, and 6 weeks following massive rotator cuff tear surgery (tenotomy and denervation), however, hematoxylin-eosin (*H&E*) staining demonstrated progressive myofiber atrophy (original magnification ×200 [**B**] and x100 [**C**, **D**]) (**B-D**), Oil Red O staining for lipids demonstrated progressive adipogenesis and lipid droplet accumulation at low (original magnification ×50 [**F**, **G**] and ×100 [**H**]) (**F-H**) and high (original magnification ×400) (**J-M**) magnifications, and Picro-Sirius Red staining for type I and type III collagen demonstrated progressive fibrogenesis (original magnification ×100) (**O-Q**) in supraspinatus tissue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tears, we tracked these cells via their GFP fluorescence in vitro after tendon injury. Digestion of the rotator cuff muscle yielded a mixture of mononuclear cells and fractured myotubes, which were further cultured without the addition of any differentiation-inducing factors. As shown in Figure 2, changes in the myogenic and fibroadipogenic potentials of rotator cuff–derived cells after injury were reflected in vitro in a time-dependent manner. At 5 days (Fig. 2, *B* and *F*) to 2 weeks (Fig. 2, *C* and *G*) after injury, we noted myogenesis of GFP$^+$ cells, while at 6 weeks (Fig. 2, *D* and *H*), myogenesis diminished and GFP$^+$ adipocytes (Fig. 2, *H*) and GFP$^+$ fibroblast-like cells (Fig. 2, *J*) increased in rotator cuff–derived cultures. This finding implies that fibroadipogenic cells originated from GFP$^+$ PDGFRβ$^+$ cells. Accordingly, quantitative PCR results (Fig. 2, *K*) coincided with the kinetics of cellular fibroadipogenesis, showing a marked increase in the expression of genes associated with induction of fibrosis (collagen III) and adipogenesis (leptin) within 6 weeks after TT-DN. These findings demonstrate a time-dependent transition in post-injury PDGFRβ$^+$ perivascular cells from predominantly myogenic to predominantly fibroadipogenic activity.

PDGFRβ$^+$ PDGFRα$^+$ cell subset resides in murine rotator cuff tissue and contributes to both fibrosis and adipogenesis after massive rotator cuff tears in vivo

To determine whether a PDGFRα$^+$ subset of PDGFRβ$^+$ cells contributes to fibrosis and adipogenesis following TT-DN surgery in vivo, we examined GFP$^+$ cells in native and experimental rotator cuff muscle tissue and analyzed the presence of PDGFRα and PDGFRβ within these tissues. On fluorescent confocal microscopy, we observed GFP$^+$ cells in uninjured

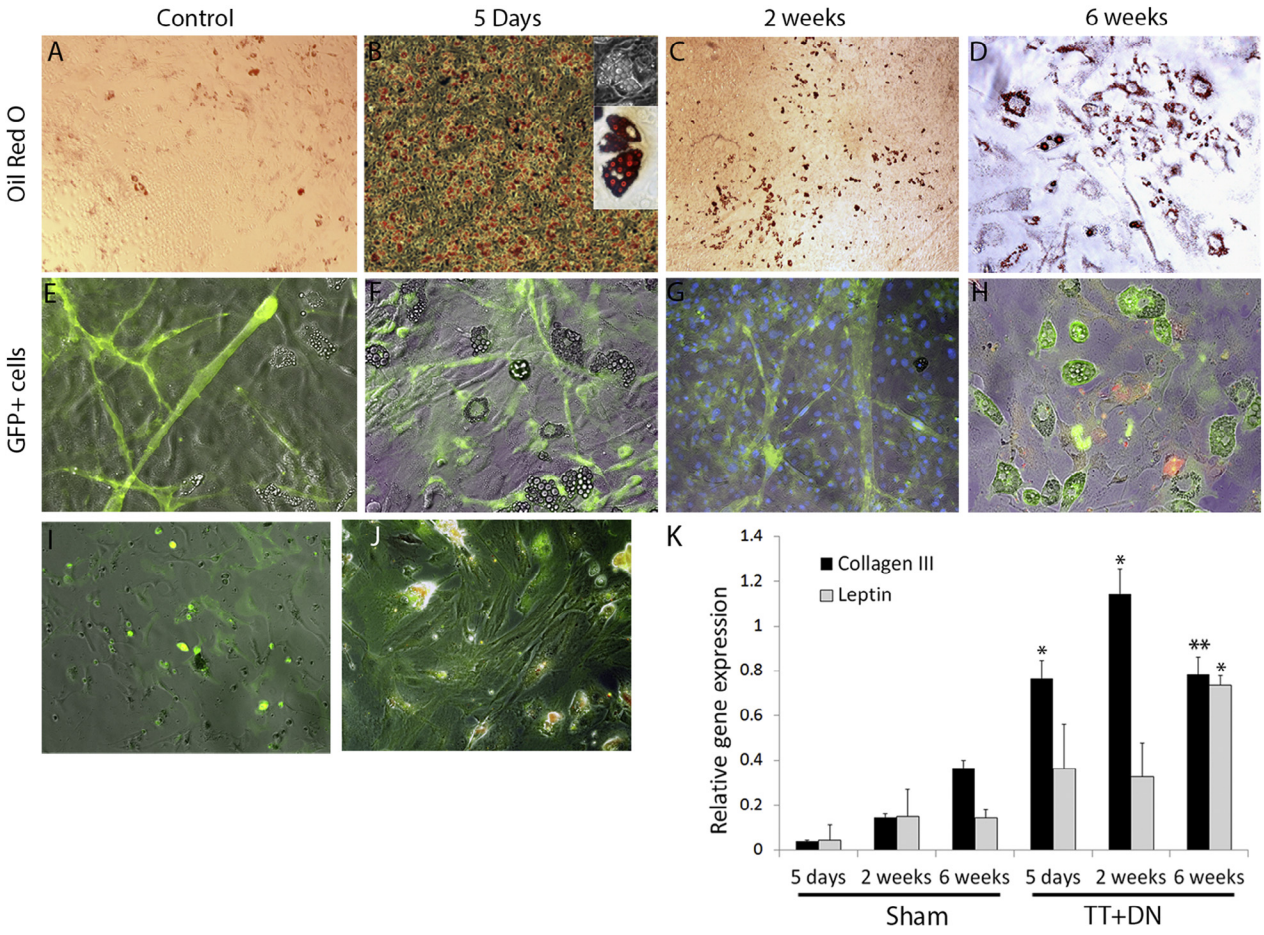

Figure 2 Rotator cuff–derived cell cultures from transgenic mice successfully mimic remodeling of injured rotator cuff in vivo at specific time points following injury. We excised rotator cuff muscle tissue from healthy uninjured (control) mice and from surgical mice at 5 days, 2 weeks, and 6 weeks after tenotomy and denervation (*TT+DN*). Cells isolated from uninjured mice were minimally adipogenic (original magnification ×40 and ×200) (**A, E**), whereas cells isolated from surgical mice at all 3 time points were highly adipogenic (original magnification ×40 [**B**, **C**] and ×200 [**D**, **F**, **G**, **H**]) (**B-D, F-H**). In comparison with the uninjured rotator cuff cell culture (original magnification ×100) (**I**), green fluorescent protein (*GFP*)–positive fibroblast-like cells dominated the rotator cuff cell cultures 6 weeks after TT+DN (original magnification ×100) (**J**). The expression of collagen III and leptin was increased in cells isolated from TT+DN mice compared with sham-surgery mice (**K**). Relative expression of fibroadipogenic genes is shown at the indicated time points after TT+DN compared with uninjured rotator cuff controls. Data are presented as mean + standard error of the mean, with 3 mice per time point. *One asterisk* indicates $P < .0001$ and *two asterisks* indicate $P < .001$ compared with matched sham controls (2-tailed Student *t* test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and sham-surgery rotator cuff tissue predominantly in the skeletal muscle interstitial spaces and incorporated into blood vessels within the perimysium (Fig. 3, *A*). At 2 weeks after TT-DN, however, we visualized GFP$^+$ adipocytes and GFP$^+$ disorganized fibrotic tissue in supraspinatus and infraspinatus muscle tissue sections (Fig. 3, *B* and *C*). At 6 weeks after TT-DN, we visualized GFP$^+$ cells within collagen bundles arranged in a parallel fashion, having adopted a thicker and more well-organized structure (Fig. 3, *C*, inset). Immunolabeling revealed that PDGFRα and PDGFRβ co-localized with GFP in fibrotic tissue (Fig. 3, *D-G*). Together, these findings suggest that a subpopulation of PDGFRβ$^+$ cells that co-express PDGFRα exhibit features of FAPs.

We confirmed the existence of such a GFP$^+$ PDGFRα$^+$ subset by flow cytometry analyses (Fig. 3, *H*) and by observing GFP$^+$ PDGFRα$^+$ cells localizing within the interstitial space of rotator cuff musculature (Fig. 3, *I*). We measured the dynamic frequency of this subset throughout the post-injury remodeling process of the rotator cuff. We noted a significant increase in GFP$^+$ PDGFRα$^+$ cell population frequency at 5 days after TT-DN, followed by a reduction in frequency to basal levels within 2 weeks after TT-DN (Fig. 3, *I* and *J*). In addition, collagen production increased with time, peaking at 6 weeks after TT-DN and mirroring GFP$^+$ cell detection in fibrotic areas of muscle sections from transgenic mice (Fig. 3, *K*). These data demonstrate that the

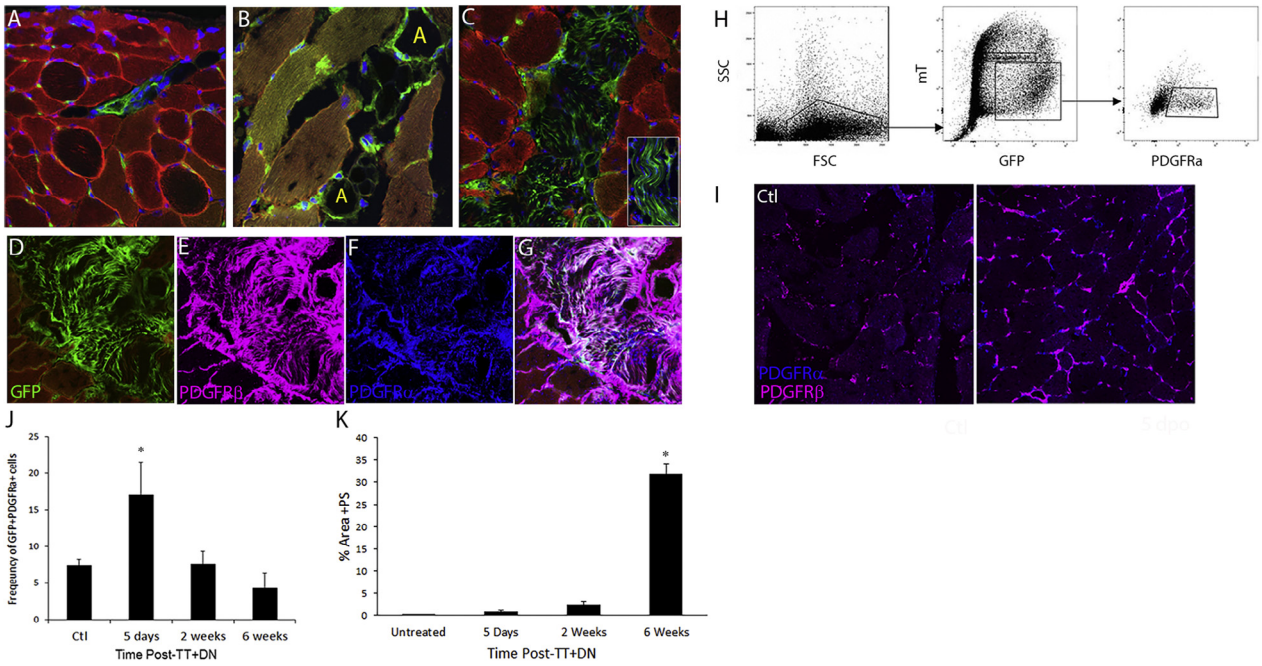

Figure 3 Platelet-derived growth factor receptor β (*PDGFRβ*)–positive platelet-derived growth factor receptor α (*PDGFRα*)–positive cells localized to fibrotic and fat tissue following massive rotator cuff tears. In uninjured mice, green fluorescent protein (*GFP*)–positive cells localized to the perivascular space (original magnification ×200) (**A**). At 2 weeks following tenotomy and denervation (*TT+DN*), adipocytes (*panel B*, *letter A*) containing lipid droplets expressed GFP (original magnification ×200) (**B**) and interstitial fibrotic tissue (original magnification ×200) (**C**). This interstitial fibrotic tissue underwent further organization by 6 weeks after TT+DN (**C**, *inset*). GFP$^+$ cells (original magnification ×200) (**D**) in the interstitial scar tissue expressed both PDGFRβ (original magnification ×200) (**E**) and PDGFRα (original magnification ×200) (**F**) on co-localization studies (original magnification ×200) (**G**). Fluorescence-activated cell sorting analysis (original magnification ×200) (**H**) demonstrated a distinct population of GFP$^+$ PDGFRα$^+$ cells residing in the rotator cuff muscle tissue. This cell population underwent expansion in vivo at 5 days after TT+DN and localized to the interstitial and perivascular tissue (original magnification ×100) (**I**). According to fluorescence-activated cell sorting analysis of digested rotator cuff muscle, the number of PDGFRβ$^+$ PDGFRα$^+$ cells significantly increased from baseline at 5 days after TT+DN and then subsequently decreased back to basal levels within 2 weeks ($P < .001$, single-factor analysis of variance) (**J**). The *asterisk* indicates that the 5-day time point was significantly greater than the control (*Ctl*), 2-week, and 6-week time points (Tukey-Kramer post hoc test). Quantification of collagen red staining demonstrated a significant increase in collagen production at 6 weeks after TT+DN ($P < .0001$, single-factor analysis of variance) (**K**). The *asterisk* indicates that the 6-week time point was significantly greater than the untreated, 5-day, and 2-week time points (Tukey HSD [honest significant difference] post hoc test). *SSC*, side scatter; *mT*, tomato red fluorescent protein; *FSC*, forward scatter; *Ctl*, control; *+PS*, picro-sirius positive. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PDGFRβ$^+$ PDGFRα$^+$ progenitor cell population contributes to fibrosis and adipogenesis of the mouse rotator cuff after massive tendon tear injury in vivo.

PDGFRβ$^+$ cells that co-express PDGFRα are fibrogenic and adipogenic in vitro

To investigate whether rotator cuff–derived PDGFRβ$^+$ PDGFRα$^+$ cells possess fibrotic and adipogenic potentials, we isolated these cells from uninjured supraspinatus and infraspinatus muscle tissue and treated them with induction media. Using FACS, we first gated GFP$^+$ cells and then sorted the GFP$^+$ PDGFRβ$^+$ PDGFRα$^+$ and GFP$^+$ PDGFRβ$^+$ PDGFRα$^-$ subsets from short-term expanded dissociated cells. We sorted cells from culture instead of directly from muscle digestions because the digestion process resulted in a transient loss of cell-surface PDGFRβ expression, which subsequently was restored within a few days in culture. We found that all PDGFRα$^+$ cells, following 3-5 days in culture, co-expressed PDGFRβ as well as GFP (Fig. 4, *A*) and maintained GFP expression after FACS sorting for up to 3 passages (Fig. 4, *B*). Sorted and induced GFP$^+$ PDGFRβ$^+$ PDGFRα$^+$ cells efficiently differentiated in vitro into GFP$^+$ adipocytes, adopting round morphology and accumulating lipids (Fig. 4, *C*). GFP$^+$ PDGFRβ$^+$ PDGFRα$^+$ cells exhibited significantly more collagen production both in non-induction media (Fig. 4, *F*, "Ctrl") and in the presence of TGF-β1 (Fig. 4, *F*, "B1") than GFP$^+$ PDGFRβ$^+$ PDGFRα$^-$ cells. On comparison of GFP$^+$ PDGFRβ$^+$ PDGFRα$^+$ in non-induction media (Fig. 4, *D*) and in TGF-β1–containing media (Fig. 4, *E*), these cells significantly increased the production of collagen, whereas there was no change in collagen production from GFP$^+$ PDGFRβ$^+$ PDGFRα$^-$ cells with TGF-β1 treatment (Fig. 4, *F*).

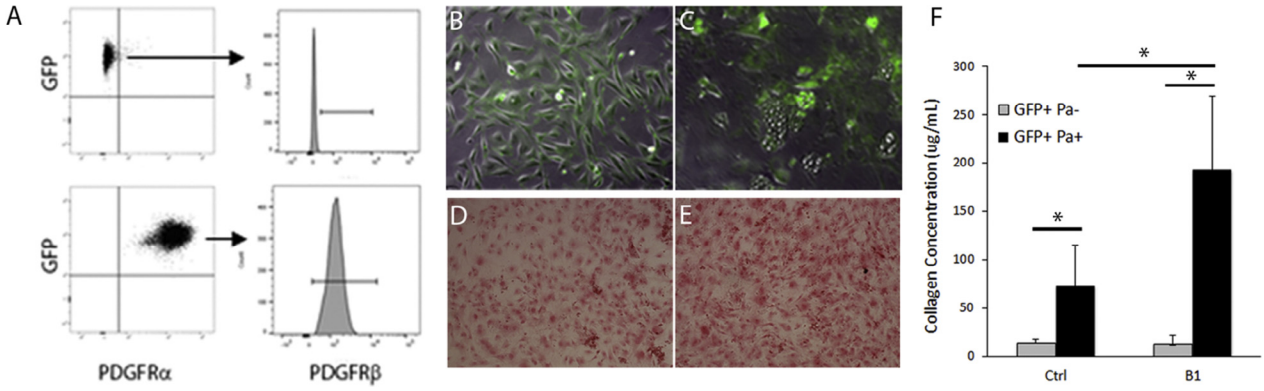

Figure 4 Platelet-derived growth factor receptor β (*PDGFRβ*)–positive platelet-derived growth factor receptor α (*PDGFRα*)–positive cells—but not PDGFRβ$^+$ PDGFRα$^-$ cells—from the rotator cuff possessed both fibrotic and adipogenic potential. Green fluorescent protein (*GFP*)–positive cells were sorted into 2 distinct populations based on PDGFRα expression in flow cytometry (**A**). When stimulated with transforming growth factor β1 (TGF-β1), the PDGFRβ$^+$ PDGFRα$^+$ cell population possessed both increased adipogenic (**B** vs **D**) and fibrotic (**C** vs **E**) potential in vitro compared with control. Collagen quantification measurements demonstrated that PDGFRβ$^+$ PDGFRα$^+$ cells expressed significantly more collagen than PDGFRβ$^+$ PDGFRα$^-$ cells with and without stimulation by TGF-β1 (*asterisk*, $P = .0053$ in control group [*Ctrl*] and $P = .0046$ in TGF-β1–treated group [*B1*] between cell subpopulations; *pound sign*, $P = .011$ between PDGFRα$^+$ PDGFRβ$^+$ experimental groups [2-tailed Student *t* test]) (**F**). (Original magnification ×100.) *Pa*, PDGFRα. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Collectively, these findings demonstrate that co-expression of PDGFRα and PDGFRβ labels a subset of progenitor cells within the rotator cuff skeletal muscle capable of adipogenic and fibrotic differentiation.

Small-molecule CWHM-12 inhibits fibrosis from PDGFRα$^+$ PDGFRβ$^+$ cell subset in vitro

CWHM-12 has been shown to efficiently inhibit fibrosis in liver, kidney, and lung tissue.[17] To investigate whether CWHM-12 was able to inhibit fibrosis specifically from the rotator cuff–derived PDGFRβ$^+$ PDGFRα$^+$ cell population in vitro, we cultured PDGFRβ$^+$ PDGFRα$^+$ cells in standard culture, in TGF-β1 induction media, and in TGF-β1 induction media treated with CWHM-12. PDGFRβ$^+$ PDGFRα$^+$ cells in standard culture media produced minimal collagen (Fig. 5, *A* and *D*), while culture in TGF-β1 induction media led to a significant increase in collagen production (Fig. 5, *A, B,* and *D*). The addition of CWHM-12 to the TGF-β1 induction media reduced collagen production by PDGFRβ$^+$ PDGFRα$^+$ cells back to control-media levels (Fig. 5, *A* and *C*). No significant difference in collagen production existed between the standard culture media and the TGF-β1 induction media treated with CWHM-12 (Fig. 5, *D*). These data indicate that CWHM-12 treatment successfully decreases fibrogenesis by the rotator cuff–derived PDGFRβ$^+$ PDGFRα$^+$ cell population in vitro.

Discussion

Researchers have identified PDGFRβ$^+$ and PDGFRα$^+$ cell populations in tissues such as murine hindlimb,[19,42] human skeletal muscle,[41] and rotator cuff[30] as distinct fibroadipogenic subsets. Recently, 2 groups have specifically evaluated the roles of PDGFRα$^+$ cells in rotator cuff fatty degeneration. Liu et al[30] found that PDGFRα$^+$ FAP cells are the major source of adipocytes in the mouse rotator cuff, while Shirasawa et al[39] found that inhibition of this cell population via a small molecule results in decreased fatty degeneration after rotator cuff tears. The findings in our study support these conclusions and, additionally, demonstrate that it is the double-positive PDGFRβ$^+$ PDGFRα$^+$ cell population that contributes to fibrosis and fatty degeneration after massive rotator cuff tears (Fig. 6).

In addition, we found that PDGFRβ$^+$ PDGFRα$^+$ cells cause fibrosis and fatty degeneration following a mechanical injury to muscle tissue. Previously, researchers had evaluated the role of these cells only after toxin-mediated muscle injury but not in a physiologically relevant model of human disease.[19,43] Deposition of fibrotic extracellular matrix differs between acute and chronic skeletal muscle injuries.[32] The regeneration of myofibers, as well as the removal of dead myofibers and collagen, is tightly coordinated with a short inflammatory phase following acute injury. Conversely, the processes of muscle degeneration, collagen deposition, and fatty degeneration are characteristic of chronic tissue injury.[32] Transient fibrosis can originate from PDGFRα$^+$ cells following acute skeletal muscle injury from cardiotoxin injection,[43] while our studies have demonstrated that this PDGFRα$^+$ cell population is the source of permanent collagen deposition following irreversible tendon injury. Compared with toxin-mediated acute injury models, our tendon transection and denervation model further demonstrates that prolonged exposure to pathologic conditions promotes excessive collagen deposition and fatty degeneration by PDGFRα$^+$ cells.

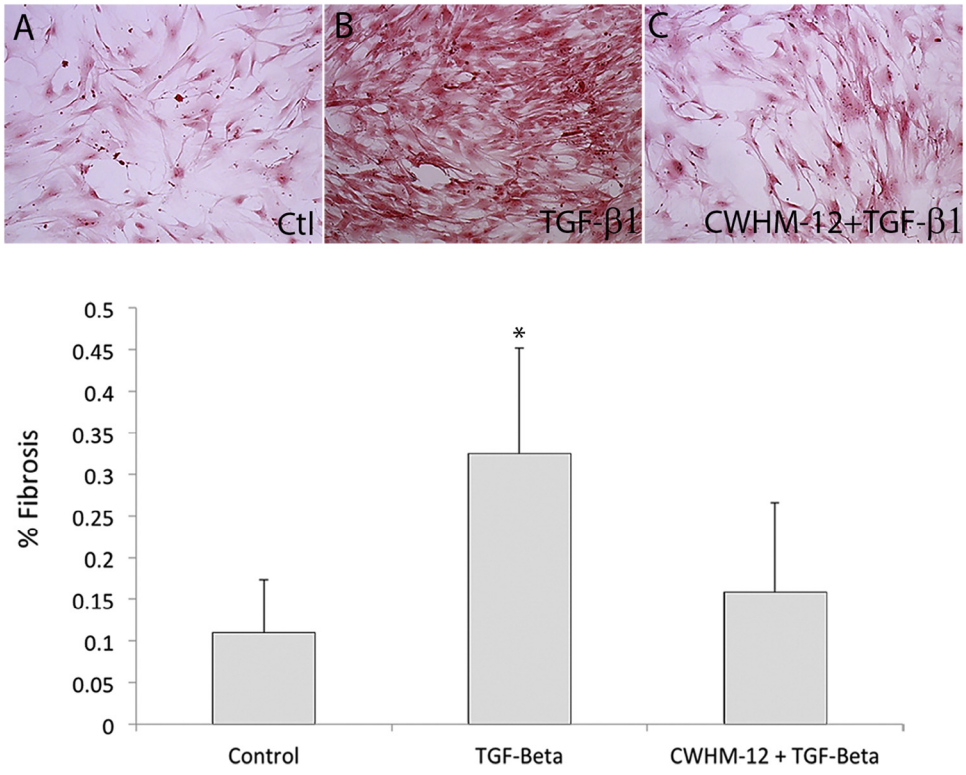

Figure 5 CWHM-12 inhibited fibrosis from the platelet-derived growth factor receptor β (PDGFRβ)–positive platelet-derived growth factor receptor α (PDGFRα)–positive cell subset. PDGFRβ$^+$ PDGFRα$^+$ cells produced minimal collagen in control media (*Ctl*) (**A**), whereas induction with transforming growth factor β1 (*TGF-β1*) led to a significant increase in fibrosis (**B**). Treatment with CWHM-12 led to a significant reduction of collagen production from PDGFRβ$^+$ PDGFRα$^+$ cells in TGF-β1 induction media (**C**). The TGF-β1 group produced significantly more fibrosis than the control and TGF-β1–plus–CWHM-12 groups, while there was no significant difference in collagen production between the control and TGF-β1–plus–CWHM-12 groups (**D**) ($P < .0001$, single-factor analysis of variance). The *asterisk* indicates that the TGF-β1 group was significantly greater than the control and TGF-β1–plus–CWHM-12 groups (Tukey-Kramer post hoc test). (Original magnification ×200.)

The clinical significance of these findings is that they identify a source of cells contributing to rotator cuff fibrosis and fatty degeneration, which is a significant predictor of poor outcomes for rotator cuff tears.[3,24] Fibrosis and fatty degeneration can lead to diminished tissue compliance, which may preclude repair and necessitate reverse total shoulder arthroplasty or tendon transfer procedures.[11,24] Understanding the role of the PDGFRβ$^+$ PDGFRα$^+$ cell population in fibrosis and fatty degeneration of the rotator cuff allows for the development of clinical strategies to target and inhibit this cell population. The ability to inhibit the causative agents of the fatty degeneration could hold significant promise in improving outcomes of surgical rotator cuff repairs. Previous studies have demonstrated that fibrotic processes are reversible when targeted on the cellular level.[17] It may be possible to reverse fibrosis of the rotator cuff musculature, as well as fatty degeneration, with the development of appropriate molecular targets used in the perioperative setting, limiting fatty degeneration and enhancing tissue regeneration.

We also demonstrated that treatment with CWHM-12 causes a decrease in the amount of fibrosis produced by PDGFRβ$^+$ PDGFRα$^+$ cells derived from murine rotator cuff tissue in vitro. This finding suggests that therapeutics can manipulate PDGFRβ$^+$ PDGFRα$^+$ cell activity to mitigate the effect of these cells on rotator cuff tissue degeneration following tears. Recently, Shirasawa et al[39] demonstrated that inhibition of PDGFRα signaling, through the administration of the tyrosine kinase inhibitor imatinib, decreased rotator cuff fatty degeneration after a massive rotator cuff tear procedure in mice. Joshi et al[23] injected rats with systemic rapamycin, a mammalian target of rapamycin inhibitor with antiadipogenic properties, following TT-DN and assessed the amount of fatty degeneration after 6 weeks. Although they did not evaluate the effect on PDGFRβ$^+$ PDGFRα$^+$ cells in particular, they noted a preservation of rotator cuff wet muscle weight, a decrease in adipogenic transcription factor production, and a decrease in histologic evidence of fatty degeneration at the rotator cuff. Ikemoto-Uezumi et al[20] used continuous systemic administration of pro-insulin-like growth factor-2, an endogenous prohormone, via a subcutaneously implanted pump to inhibit fatty degeneration after cardiotoxin injection in mice hindlimbs. They found that this treatment successfully decreased fatty degeneration on histologic analysis and that in vitro exposure of PDGFRα$^+$ cells to sufficient

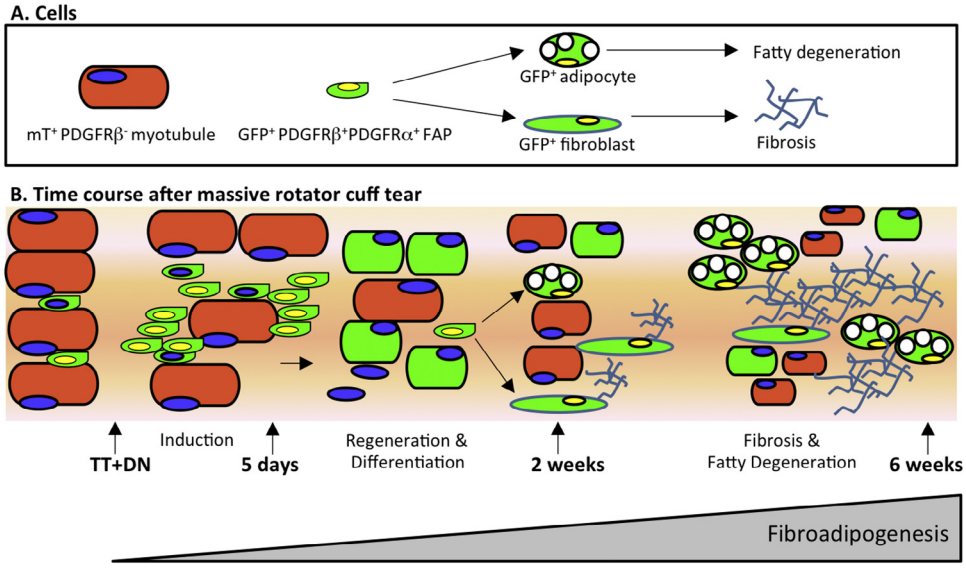

Figure 6 Summary diagram of cellular changes following tenotomy and denervation (*TT+DN*) (of supraspinatus and infraspinatus muscles) in rotator cuff of mouse model. Platelet-derived growth factor receptor β (*PDGFRβ*)–negative myotubules are red under fluorescence whereas PDGFRβ+ cells are green due to the mTmG Cre-Lox system (**A**). In uninjured rotator cuff muscle tissue, red myotubules dominate and green fluorescent protein (*GFP*)–positive fibroadipogenic progenitor (*FAP*) cells are rare and quiescent (**B**). After massive rotator cuff tear (TT+DN), the GFP+ FAP cells, which also express PDGFRβ and platelet-derived growth factor receptor α (*PDGFRα*), are induced and undergo differentiation into GFP+ adipocytes and GFP+ fibroblasts. These cells ultimately cause degenerative tissue changes within 6 weeks of the rotator cuff tear. *mT*, tomato red fluorescent protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

concentrations of pro-IGF2 inhibited this cell population's differentiation into adipocytes. Other therapeutics, such as anabolic steroids[13] and oral tamoxifen,[6] have been found to decrease muscle atrophy but have had no effect on fatty degeneration in animal models of massive rotator cuff tears. Collectively, these experiments show that pharmacologic intervention may be able to improve rotator cuff tissue regeneration and that pro-IGF2 in particular may inhibit PDGFRβ+ PDGFRα+ cell adipogenic differentiation. Further experiments should be conducted to confirm whether mTOR inhibitors and pro-IGF2 can diminish the adipogenic differentiation of PDGFRβ+ PDGFRα+ cells in the setting of massive rotator cuff tendon tears.

Instead of inhibition of degradative pathways, other investigators have tested the augmentation of myogenic pathways following massive rotator cuff tears in an attempt to improve tissue regeneration.[10,16] Deciphering the diversity of muscle precursor populations, such as PDGFRβ+ pericytes, is an important consideration for these translational myogenic regeneration programs. The PDGFRα+ subpopulation of PDGFRβ+ cells, for instance, appears to have minimal myogenic capacity and instead play a role in the degenerative processes of muscle fibrosis and fatty degeneration.

PDGFRβ+ PDGFRα+ cells also provide a supportive role in muscle tissue regeneration in certain environmental conditions, as researchers have found them to clear necrotic debris in healthy, regenerating muscle.[19,22] These findings have clinical significance in other degenerative muscle conditions, such as muscular dystrophy[4,8,46] and age-associated sarcopenia,[20,32] fields in which regenerative medicine is particularly relevant. Elucidating this complex interaction between PDGFRβ+ PDGFRα+ and other cell populations and the effect of the external cellular milieu is important for the further development of clinical protocols promoting muscle regeneration.

PDGFRβ+ PDGFRα+ cells may be derived from other cell populations such as myofibroblasts, which are cells localized to the perivascular space that express PDGFRβ.[15] In fibrotic disease models of the lung, liver, heart, and kidney, PDGFRβ expression strongly correlated to blood vessel–residing pericytes that differentiate into myofibroblasts on activation of PDGFRβ and increased matrix synthesis.[12] Coinciding with our observations, a subset of human lung PDGFRβ+ ATP-binding cassette sub-family G member 2+ pericytes exhibited increased expression of PDGFRα in pulmonary fibrosis and differentiated in vivo into alpha smooth muscle Actin+ myofibroblasts during degenerative remodeling associated with bleomycin-induced pulmonary fibrosis in a murine experimental model.[33] In another animal model, overexpression of PDGFRα was shown to lead to systemic fibrosis.[35] It is unclear whether the expression of PDGFRα alone, without PDGFRβ, represents a distinguished subset with similar fibroadipogenic features or the same FAP cell subpopulation and whether these subsets exist and exhibit similar fibroadipogenic characteristics in other tissues.

Successful therapeutic intervention should result in restoration of balanced healthy-tissue signaling pathways as well as cell composition and phenotype. Mutual functional interactions between myogenic progenitor cells and skeletal

muscle–residing non-myogenic cells regulate skeletal tissue maintenance in the steady state via cell-cell interactions and secreted factors.[14] Therefore, the elucidation of rotator cuff subpopulations will be instrumental in the development of cell therapy–based muscle regeneration strategies. Transplantation of myogenic precursor cells, with PDGFRβ$^+$ PDGFRα$^+$ cells in a myo-supportive rather than fibroadipogenic state, may replenish injured tissue with healthy muscle cells. However, injury-induced signaling pathways may promote graft failure and further shift back the phenotype of PDGFRβ$^+$ PDGFRα$^+$ cells from undifferentiated progenitors into a fibroadipogenic state. Administration of drugs that inhibit pro-fibroadipogenic signaling pathways, such as myostatin and activin receptors,[40] could therefore be combined with cell therapy to achieve the most effective treatment. Supporting this combinatory therapeutic concept, it has been demonstrated in the diabetes research literature that a combination therapy of human embryonic stem cell–derived pancreatic precursors and an antidiabetic drug is more effective than either progenitor cell transplant or antidiabetic drug administration alone.[2]

Patients with fatty degeneration of the rotator cuff experience muscle degeneration and poor clinical outcomes following nonoperative and operative treatment.[24,31] Understanding the etiology of this fatty degeneration, the interaction of PDGFRβ$^+$ PDGFRα$^+$ cells with the local environment, and methods of optimizing myogenic regeneration will hopefully lead to clinical protocols that may be able to prevent or reverse fatty degeneration and, consequently, improve patient outcomes.

Limitations

As with other animal studies, differences exist between the anatomy and physiology of the mouse rotator cuff and the human rotator cuff. Furthermore, while the TT-DN model does reproduce the pathologic changes seen in the human rotator cuff, it is ultimately based on an acute tendon tear and a neurologic injury mechanism instead of a more clinically relevant chronic injury model. Future experiments should be conducted in an animal model based on chronic cuff tears in aged animals to further recapitulate human disease. Despite these differences, we believe this model represents the best approximation of human pathology based on the histopathologic muscle degeneration in our and other groups' studies.[26-29,37] We used a mouse model with a non-inducible Cre system, and therefore, all mouse cells that expressed PDGFRβ at some point in their lineage also expressed GFP, whether or not PDGFRβ remained present. We accounted for this limitation by confirming the presence of PDGFRβ on GFP$^+$ cells on both immunohistochemistry (Fig. 3, *D* and *E*) and flow cytometry (Fig. 4, *A*). Still, the use of an inducible system, while presenting limitations of its own, would have circumvented the need for this PDGFRβ confirmation step. In addition, although PDGFRβ$^+$ PDGFRα$^+$ progenitor cells have been identified in human skeletal muscle,[41] their role in human pathology, as opposed to the mouse model, remains to be fully understood. Last, selective depletion of α_v integrins on PDGFRβ$^+$ PDGFRα$^+$ cells will further confirm their functional contribution to TGF-β1–mediated fibrosis.

Conclusions

Our findings demonstrate that PDGFRβ$^+$ PDGFRα$^+$ cells directly contribute to muscle fibrosis and fatty degeneration in a mouse model of massive rotator cuff tears. Further studies are required to confirm the role of these cells in fibrosis and fatty degeneration of the rotator cuff in the human shoulder. The perioperative inhibition of the PDGFRβ$^+$ PDGFRα$^+$ cell population may represent an attractive target for the prevention or reversal of the fibroadipogenic changes that are associated with large rotator cuff tears. Further research is required to evaluate the role of such therapies.

Disclaimer

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