Differences in transcriptional patterns of extracellular matrix, inflammatory, and myogenic regulatory in myofibroblasts, fibroblasts, and muscle precursor cells isolated from old male rat skeletal muscle using a novel cell isolation procedure

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Abstract Aged skeletal muscle displays increased fibrosis and impaired regeneration. While it is not well characterized how skeletal muscle fibroblasts contribute to these phenomena, transforming growth factor- *b*1 (TGF-*b*1) and Delta/Notch signaling have been implicated to influence muscle regeneration. In this study, a unique combination of aging phenotypes is identified in differentiating fibroblasts (myofibroblasts), proliferating fibroblasts, and muscle precursor cells (MPCs) that characterize an impaired regenerative potential observed in aged skeletal muscle. Using a novel dual-isolation technique, that isolates fibro- blasts and MPCs from the same rat skeletal muscle sample, and cell culture conditions of 5 % O_2 and 5 % CO_2, we report for the first time that myofibroblasts from 32-mo-old skeletal muscle, compared to 3-mo- old, display increased levels of mRNA for the essential extracellular matrix (ECM) genes, collagen 4*a*1 (83 % increase), collagen 4*a*2 (98 % increase), and laminin 2

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(113 % increase), as well as increased levels of mRNA for the inflammatory markers, interleukin-6 (4.3-fold increase) and tumor necrosis factor *a* (3.2-fold increase), and TGF-*b*1 (84 % increase), whose protein controls proliferation and differentiation. Additionally, we demonstrate that proliferating fibroblasts from 32-mo-old skeletal muscle display increased levels of mRNA for the Notch ligand, Delta 1 (C2.0-fold increase). Together, these findings suggest that increased expression of ECM and inflammatory genes in myofibroblasts from 32-mo-old skeletal muscle may contribute to the fibrogenic phenotype that impairs regeneration in aged skeletal muscle. Furthermore, we believe the novel dual-isolation technique developed here may be useful in studies that investigate communications among MPCs, fibroblasts, and myofibroblasts in skeletal muscle.

Keywords Aging · Muscle · Satellite cell · Fibroblast · Fibrosis · Cell culture

Introduction

Pathological fibrosis, or the excess accumulation of extracellular matrix (ECM) components (e.g. collagen), involves the integration of many different cell types, growth factors, inflammatory and fibrogenic cytokines, and proteolytic enzymes; which together alter the tissue microenvironment and stimulate the overproduction of connective tissue elements, ultimately remodeling the normal tissue architecture and impairing function (Mann et al. 2011). Aging is associated with an overall reduction in skeletal muscle function and mass (known as sarcopenia), an impaired capacity for skeletal muscle hypertrophy, regeneration and repair (Marsh et al. 1997; Pattison et al. 2003a) beginning at varying ages between 65 and 80 years old (Aagaard et al. 2010), and a concomitant increase in skeletal muscle fibrosis and ECM composition (Golds- pink et al. 1994). Multiple factors, including (but not limited to) the muscle fiber niche, extracellular matrix, muscle precursor cells (MPCs, which include satellite cells), microvasculature, loss of motor nerves and individual muscle fibers (motor units), immune/ inflammatory, hormonal, fibro-adipogenic cells, myokines, oxidative stress, energy/protein metabolism, and systemic factors, have been implicated in contributing to the development of this aged skeletal muscle phenotype (Gopinath and Rando 2008; Jang et al. 2011). The multiplicity of contributing factors makes it unlikely that all of these factors can be controlled, or manipulated, in a single set of experiments. Therefore, the current study focuses on characterizing three cell types (MPCs, fibroblasts, and myofibroblasts) isolated from the same skeletal muscle sample from 3- and 32-mo-old rats.

Aged skeletal muscle has impaired regenerative potential, likely due to increased fibrogenic and decreased myogenic capabilities. Brack et al. (2007) demonstrated that increased collagen deposition in regenerating skeletal muscle of aged mice was due to the conversion of myogenic cells to a fibrogenic lineage during the regeneration process. Myofibroblasts, a diverse and highly plastic type of cells that, depending on the tissue, originate from fibroblasts, and could also play a negative role in the regeneration process. Despite myofibroblasts possessing both myogenic and non-myogenic properties by expressing structural, regulatory, and contractile proteins, they do not morphologically differentiate into multinucleated myotubes, nor do they withdrawal from cell cycle. More importantly, myofibroblasts are predominately responsible for the secretion of ECM components during tissue remodeling (Walker et al. 2001). While myofibroblasts are important in normal tissue development, homeostasis, and wound repair, the molecular and cellular properties of myofibroblasts in aged skeletal muscle have not been well elucidated. Given the importance of MPCs, fibroblasts, and myofibroblasts in skeletal muscle regeneration, it is essential to examine all three cell- types and characterize them in aged skeletal muscle. A method that extracts both MPCs and fibroblasts from the same tissue of the same animal would be of great importance to help characterize the microenvironment of aged skeletal muscle and identify sources of potential inhibitors of muscle regeneration with aging.

Transforming growth factor-*b*1 (TGF-*b*1) and Delta/Notch signaling are important regulators of regenerative processes in many tissues, including skeletal muscle (Conboy et al. 2003; Conboy and Rando 2002). Delta is an extracellular ligand for the cell surface Notch receptor, thus comprising a ‘‘juxtacrine’’ signaling system whereby Delta ligands on one cell can activate Notch receptors and signaling on another when the cells are in close proximity. The expression of both Notch and Delta are decreased with age and Notch signaling is impaired with

advanced age (Carey et al. 2007; Conboy et al. 2005). Furthermore, TGF-*b*1/p-SMAD3 signaling antagonizes Notch signaling (Carlson et al. 2008). TGF-*b*1 is thought to mediate at least some of the age- associated defect in muscle regeneration via auto/ paracrine or endocrine mechanisms. Elevated levels of TGF-*b*1 protein have been reported in serum and the interstitial space surrounding muscle fibers in old mice, relative to young (Carlson et al. 2008, 2009). It is unknown however, whether the muscle interstitial TGF-*b*1 is produced locally or systemically; and if produced locally, by what cell types contained within the muscle tissue. Interestingly, while TGF-*b* super- family members antagonize proliferative signaling in MPCs, they stimulate proliferation and protein syn- thesis in fibroblasts (Li et al. 2008; Duncan et al. 1999). Taken together, this evidence suggests that the age-related impairment in muscle regeneration could be also
be regulated by skeletal muscle fibroblasts and/or myofibroblasts.

Therefore, the aims of the present study were to: first, develop a dual-isolation procedure for the extraction of MPCs and skeletal muscle fibroblasts from the same skeletal muscle tissue sample; and second, to investigate whether differences in mRNA levels exist in MPCs, fibroblasts, and myofibroblasts isolated from the same sample of skeletal muscle from 3- and 32-mo-old rats.

Experimental procedures

Animals

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri-Columbia. Young (3-month-old; -mo-old) and old (32-mo-old) Fischer-344 x Brown Norway F_1 hybrid male rats were obtained from the National Institute on Aging. These ages were selected for their growth and skeletal muscle mass characteristics, such that 3-mo-old are young, growing rats and 32-mo-old are old, sarcopenic rats, according to growth curves (Turturro et al. 1999) and muscle weights (Lushaj et al. 2008), previously reported for Fischer 344 x Brown Norway F_1 hybrid male rats. Animals were housed at 21 °C on a 12:12-h light–dark cycle and allowed free access to food and water. At the time of sacrifice, animals were given an intraperitoneal injection of ketamine (80 mg kg^{-1}), xylazine (10 mg kg^{-1}), and acepromazine (4 mg kg^{-1}), and muscle tissues of both hindlimbs were then excised and stripped of connective, adipose, and vascular tissues.

Dual cell isolation procedures and culture conditions

Muscle precursor cells (MPCs) and skeletal muscle fibroblasts were isolated from the same muscle tissue samples of either 3- or 32-mo-old rats (n = 4 rats/age group) using subsequent, but separate procedures (see Fig. 1 for diagram). First, MPCs (which include satellite cells) were isolated using the method developed by Allen et al. (1997), incorporating the modifications made by Lees et al. (2009). The quadriceps or gastrocnemius and plantaris muscles were minced on ice with minimal volumes of sterile phosphate-buffered saline (PBS). MPCs were isolated from muscle tissues using pronase digestion [1.25 mg mL^{-1} of protease type XIV (Sigma; P5147) in sterile PBS] by incubating the muscle:pronase slurry at 37 °C for 1 h with gentle agitation. The digested muscle slurries were then centrifuged at 1,500*g* for 5 min. The digested muscle pellet was resuspended in 20 mL of warm, sterile PBS, triturated 10 times to liberate the MPCs, and centrifuged at 500*g* for 10 min. The resulting supernate (containing MPCs) was collected in a new tube. The resuspension, trituration, and centrifugation steps were repeated for a total of 3 times, reducing centrifugation time in each subsequent step from 10 to 8, and finally 1 min, respectively. The remaining muscle fragment pellet contained the skeletal muscle fibroblasts (see below). The collected supernates were filtered through a 100 lm cell stainer and then centrifuged at 1,500*g* for 5 min to pellet the MPCs. The MPC pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM; containing 10 % horse serum, 100 U mL^{-1} penicillin, 100 lg mL^{-1} streptomycin, and 40 lg mL^{-1} gentamicin) and pre-plated for 24 h on tissue culture-treated 150 mm plates. The pre-plating procedure is necessary to minimize non-myogenic cell contamination because myogenic cells do not readily attach to standard cell culture-treated plates (Blau and Webster 1981). After 24 h, the pre-plates were gently agitated and the pre-plate media (containing non-adherent myogenic cells)

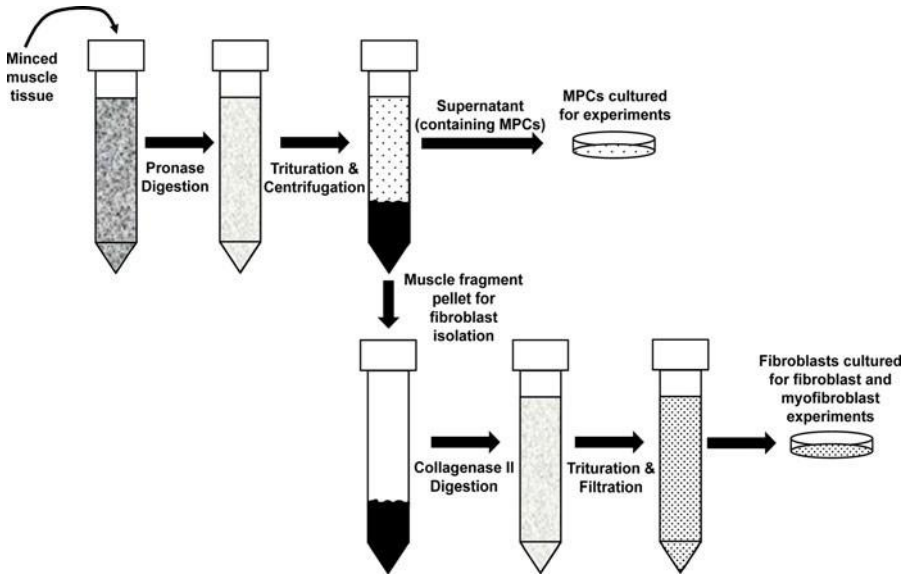

Fig. 1 Muscle precursor cells (MPCs) and skeletal muscle fibroblasts were isolated from the same sample of rat muscle tissue using subsequent, but separate procedures as diagrammed in Fig. 1. First, muscle tissue was minced in sterile PBS and digested in protease type XIV (pronase) enzymes. MPCs were liberated from muscle tissue by multiple steps of trituration and centrifugation, and then cultured for MPC experiments. Next, the remaining muscle fragment pellet was digested with collagenase II enzymes and fibroblasts were isolated with multiple steps of trituration, filtration, and centrifugation. Isolated fibroblasts were cultured for fibroblast and myofibroblast experiments. See ''Experimental procedures'' section for a detailed description of the dual cell isolation procedures

was collected, then plates were rinsed with warm pre-plate media to remove any remaining MPCs from the pre-plate. MPCs were centrifuged at 1,500*g* for 5 min, resuspended in MPC growth media (MPC–GM; 20 % FBS, 100 U mL^{-1} penicillin, 100 lg mL^{-1} streptomycin and 40 lg mL^{-1} gentamicin in Ham's F-10), aliquoted and frozen in 5 % dimethyl sulfoxide/growth media. All cell culture experiments were performed in a humidified incubator with 5 % O_2 and 5 % CO_2 at 37 °C (HERAcell, Thermo Scientifi). Our laboratory previously reported that 5 % O_2 enhances BrdU incorporation of MPCs, compared to 21 % O_2 (Lees et al. 2008b), likely because 5 % O_2 more closely represents physiological gas partial pressures than ambient oxygen conditions (Csete 2005).

Skeletal muscle fibroblasts were then isolated from the remaining muscle fragment pellets (from above) after MPC isolation. The muscle fragment pellets were resuspended in 10 mL of 1,000 U mL^{-1} collagenase II (Worthington Biochemical Corporation), mixed by thorough trituration and incubated for 1–2 h at 37 °C with gentle agitation (until solution was homogenous and free of particular matter). Next, fibroblast growth media (FB–GM; 10 % FBS in DMEM) was added up to 30 mL and the solution was filtered through a 100 lm cell strainer. The filtered solution was centrifuged at 500*g* for 5 min, the supernate discarded, and the pellet resuspended in 10 mL of FB–GM. After filtration, the resuspension, centrifugation, and supernate discard steps were repeated for a total of 4 times, but subsequent centrifugations were performed at 300*g* for 1 min. After the last supernate discard, the remaining fibroblast cell pellet was resuspended in 20 mL of FB–GM and seeded onto a 150 mm cell culture-treated plate. Media was changed at 2 and 24 h post-seeding on all fibroblast culture plates. All fibroblast experiments were performed immediately after isolation, thus omitting the freeze/thaw process.

Immunocytochemistry

MPCs and fibroblasts for immunocytochemistry were isolated as described above and for most stainings

were passaged only once. Proliferating MPCs and fibroblasts were seeded at *60 % confluency and grown for 24 h before fixation and staining; whereas differentiated fibroblasts were seeded at *90 % confluency, grown for 24 h in FB–GM and then changed to differentiation media (2 % HS/DMEM) for 3 days before fixation and staining. Except for the co-culture experiments of MPCs and fibroblasts (described in Supplemental Materials), all cells were seeded by individual cell type in either chamber slides (Nunc Lab-Tek Permanox 2 chamber slides) or in Corning 100 mm culture dishes (with coverslips added for mounting).

For fixation, cells were rinsed in PBS and incubated with either 100 % methanol at -20 °C for 10 min or 3.7 % formaldehyde at room temperature in PBS for 10 min, in both cases followed by another PBS rinse and antibody incubation.

Desmin primary antibody was incubated for 60 min in 0.1 % Triton X-100 PBS with 1.5 % Normal Goat Serum (NGS). Both smooth muscle actin-FITC conjugated (Green) antibody and the Alexa Fluor 568 (for identification of MPCs; Red) anti-rabbit secondary antibody were incubated in 1.5 % NGS PBS for 60 min. DAPI stain was applied last for 5 min for nuclear staining (Blue). Antibody identities, concentrations and their respective fixation types are described in Table 1.

All micrographs were taken using an Olympus IX70 inverted microscope with Hamamatsu ORCA-AG deep-cooled CCD camera at the molecular Cytology Core, University of Missouri, Columbia, with appropriate filters (Chroma Technology Corporation).

Table 1 Antibodies and conditions used for immunocytochemistry

Primary antibodies	Manufacturer	Catalog #	Fixing	Dilution
MyoD	Santa Cruz	Sc-760	Formalin	1:200
Desmin	Santa Cruz	Sc-14026	MeOH	1:50
Vimentin	Santa Cruz	Sc-6260	MeOH	1:200
a-Smooth muscle actin	Sigma	F3777	MeOH	1:250
Collagen I	Cell Sciences	PS041	Formalin	1:200
Myosin heavy chain	DSHB	MF-20	MeOH	1:200
CD31	Santa Cruz	Sc-1506	MeOH	1:200

DSHB Developmental Studies Hybridoma Bank

BrdU incorporation experiments

Frozen, zero passage MPC aliquots, isolated from quadriceps muscles, were thawed in a 37 °C water bath and immediately seeded onto 100 mm Matrigel-coated (0.01 mg mL^{-1} Matrigel, 60 min at 37 °C) plates in MPC growth media (MPC–GM; 20 % FBS, 100 U mL^{-1} penicillin, 100 lg mL^{-1} streptomycin and 40 lg mL^{-1} gentamicin in Ham's F-10). At 60–70 % confluency (*3 days of growth), cells were passaged to Matrigel-coated 100 mm Corning plates with antibiotic-free growth media (1.5 mL/well, 20 % FBS/Ham's F-10) at a density of 150 K cells/plate. BrdU incorporation was measured as an index of the state of proliferation (Hall and Levison 1990) in MPCs at 24 and 48 h post passage, as described previously (Lees et al. 2008a). Briefly, MPCs were pulsed with 10 lM of BrdU for 1 h at 37 °C in a humidified incubator, cultures were trypsinized, then fixed with ice-cold 70 % EtOH and stored at 4 °C until further analysis. Immunodetection of BrdU using flow cytometry was performed as described previously (Lees et al. 2008a). Cells were incubated in 2 M HCl for 30 min, followed by two neutralizing washes with 0.1 M sodium borate (pH 8.5) and a final wash in Dulbecco's phosphate-buffered saline (PBS). Cells were incubated with anti-BrdU–fluorescein (5 lg mL^{-1} in 0.1 % bovine serum albumin in PBS; Roche Applied Sciences, Indianapolis, IN, USA) for 30 min. At least 20 K cells were analyzed using a BD FAC-Scan and CellQuest Pro software (BD Biosciences), and the proportions of BrdU-positive ($BrdU^{?}$) to BrdU-negative ($BrdU^{-}$) nuclei were determined as described previously (Lees et al. 2008a).

For an estimation on the state of proliferation in skeletal muscle fibroblasts, fibroblasts were seeded after isolation in 10 mL of FB–GM on cell culture-treated 100 mm plates, grown until 60–70 % confluency with daily media changes, passaged and seeded at 150 K cells/well on 100 mm plates in 10 mL of FB–GM. BrdU incorporation was measured as an index of the state of proliferation of fibroblasts at 24 and 48 h post-passage, as described above for MPCs.

Gene expression experiments

Proliferating passage 1 (P1) MPCs and skeletal muscle fibroblasts, dual-isolated from combined gastrocnemius and plantaris muscles, were seeded separately,

by individual cell type, at 75 K cells/well in either 3 mL of MPC–GM onto Matrigel-coated 60 mm plates (0.01 mg mL^{-1} Matrigel, 60 min at 37 °C), or 3 mL of FB–GM onto cell culture-treated 60 mm plates, respectively. Cells were harvested and lysed in 300 lL of RLT buffer (with *b*-mercaptoethanol added; Qiagen) at 24 and 48 h after seeding, using QIAShredder columns (Qiagen), according to manufacturer specifications, and immediately stored at -80 °C until further analysis.

Differentiated skeletal muscle fibroblasts (myofibroblasts)

Skeletal muscle fibroblasts, isolated from the same gastrocnemius and plantaris muscles as MPCs, were seeded on a 60 mm plate (Corning) immediately after isolation and grown to *60 % confluency and then changed to differentiation media (DM, 2 % HS, DMEM). Fibroblasts had media changes 24 and 48 h later and were harvested 96 h later for RNA and lysed as described for MPCs.

RNA isolation and real-time PCR

Total cellular RNA was isolated from frozen cell lysates using the RNEasy Plus Micro kit (Qiagen), according to manufacturer specifications. Quantification of RNA was performed on each sample using spectrophotometry by measuring the absorbance at 260 nm using a Nanodrop 2000 (Thermo Scientific). RNA purity was determined by a 260:280 nm ratio of C2.0; absence of organic contamination was verified by a 230:260 nm ratio of C1.8; and RNA integrity was verified by the appearance of dense 18S and 28S rRNA bands without streaking via agarose gel electrophoresis and ethidium bromide staining. Reverse transcription was immediately performed on 1 lg of total RNA from each sample (without freeze–thaw) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer specifications. Levels of target mRNA were determined using quantitative real-time PCR (qPCR) using SYBR green PCR Master Mix (Applied Biosystems) using an ABI Prism 7000 (Applied Biosystems) with 25 ng of cDNA and 900 nM of each primer in 25 lL reactions volumes according to manufacturer specifications. Samples were performed on qPCR in triplicate on opaque 96-well plates sealed with optically-clear, heat-sealed film (Applied Biosystems) and normalized to the 18S rRNA reference gene. All primer pair sequences were generated using Primer Express® Software v2.0 from Applied Biosystems (Table 2) and specifity for target mRNA validated using BLAST (NCBI). Preliminary studies revealed that 18S rRNA levels were not different in MPCs or fibroblasts between 3- and 32-mo-old cell populations. Target mRNA levels were calculated using the DDCt method and expressed relative to young (3-mo-old) groups. All qPCR procedures meet or surpass The MIQE Guidelines (Bustin et al. 2009).

Statistical analyses

Data from the BrdU incorporation and gene expression experiments were analyzed for differences between age groups at each individual time point using Student's *t* tests. Data are reported as mean ± SEM. Significance level was set at P B 0.05.

Results

Primary tissue culture cell identification/validation

To the best of our knowledge, there are no published reports that describe a protocol for co-isolating MPCs and fibroblasts from the same skeletal muscle tissue sample and then identifying transcriptional patterns of each cell type. Therefore, one of the aims of this study was to establish a primary tissue culture model for fibroblasts isolated from the same muscle tissue from which MPCs were isolated. To validate our MPC and fibroblast dual cell co-isolation techniques, we performed immunocytochemistry for several different proteins to identify proliferating MPCs and fibroblasts in culture (Fig. 2a). While there are many viable markers used to identify satellite cells, there is no one marker that will identify all satellite cells in the tissue (Charge and Rudnicki 2004); therefore we identified myogenic cells as being positive for both MyoD and desmin staining. Additionally, these data support previous studies from our laboratory that reported [90 % MyoD- and desmin-positive cells using a MPC single-isolation technique (Machida and Booth 2004; Lees et al. 2008a, b, 2009).

Table 2 Primer sequences, melting temperatures, Fwd primer start site, and amplicon length for qPCR

mRNA target	NCBI accession number	Direction	Primer sequence (5′>3′)	T_m	Fwd primer start site (relative to ?1 ATG)	Amplicon length
TGF-*b*1	NM_021578.2	Fwd	AAACGGAAGCGCATCGAA	59	269	60
		Rev	ACTGGCGAGCCTTAGTTTGG	58		
Delta 1	NM_032063.1	Fwd	CTGCACTGACCCCATTTGTCT	59	946	62
		Rev	CCGGTTTGTCACAATATCCATGT	59		
Delta 3	NM_053666.1	Fwd	CCTGACTCTGCCTATGTCTGTCA	58	1,021	70
		Rev	GGTCCACCCTCCTCTCACAGT	59		
Delta 4	NM_001107760.1	Fwd	AGAATAGCGGCAGTGGTCGTA	58	596	62
		Rev	GGTTCCCGGCCAGGTAAA	59		
IL-6	NM_012589.1	Fwd	CCCACCAGGAACGAAAGTCA	60	19	62
		Rev	GCGGAGAGAAACTTCATAGCTGTT	63		
TNF*a*	NM_012675.3	Fwd	CCCAGAAAAGCAAGCAACCA	60	58	59
		Rev	CCTCGGGCCAGTGTATGAGA	60		
Collagen 4*a*1	NM_001135009.1	Fwd	TGCGCAAGTTCAGCACCAT	60	4,683	62
		Rev	GGCGAAGTTGCAGACGTTGT	60		
Collagen 4*a*2	XM_225043.5	Fwd	CACCAGGTGTACCCAACAATAGC	59	5,723	86
		Rev	TTCTAGGACAAGACTGAAGGAAGCT	58		
Laminin 2	XM_219866.5	Fwd	TGAGGTCATCTGCACTTCATTTTATT	59	582	91
		Rev	AGCACTTGGTCTCCCATTGATC	59		
18S rRNA	NR_046237.1	Fwd	GCCGCTAGAGGTGAAATTCTTG	59	953	66
		Rev	CATTCTTGGCAAAGCTTTCG	59		

As previously described by Li et al. (2008), we characterized fibroblasts by the following criteria: cells were considered fibroblasts by the absence of both myogenic markers (MyoD and desmin) and smooth muscle cell markers [*a*-smooth muscle actin (*a*-SMA)], but positive staining for vimentin and collagen I. The results in Fig. 2a demonstrate our ability to isolate and culture a fibroblast-enriched cell population from rat skeletal muscle tissue, simultaneously with the isolation of MPCs, prior to their differentiation, from the same animal.

In addition, we performed immunocytochemistry on differentiated cell cultures for the identification of myogenic and fibrogenic properties. According to Hinz et al. (2007), when fibroblasts are subjected to low serum conditions, they differentiate into ‘‘myofibroblasts” and begin producing scaffolding and ECM proteins, such as *a*-SMA and collagen. The expression of these proteins eventually leads to the production of fibrous tissue, thus differentiating fibroblasts will be termed myofibroblasts.

Differentiated MPCs (myotubes) were positively identified by the expression of myosin heavy chain protein (MyHC); while myofibroblasts were identified by the absence of MyHC, but relatively high expression of *a*-SMA and collagen I (Fig. 2b). Also, our putative myofibroblast population appeared visibly ‘‘stringy’’ as would be expected for load-bearing connective tissue cells. Upon serum-deprivation, myofibroblasts produce scaffolding and extracellular matrix (ECM) proteins as evident from visual inspection and previous reports elsewhere (Duncan et al. 1999; Frazier et al. 1996). With prolonged differentiation of fibroblasts, we observed changes in the viscosity of culture media supernatant (data not shown). This observation is likely caused by the presence of secreted proteins into the media as has been described previously in fibroblast culture systems (Eickelberg et al. 1999).

Adult rat primary smooth muscle cells and endothelial cells were obtained from other laboratories and immunocytochemistry was performed to use these cell

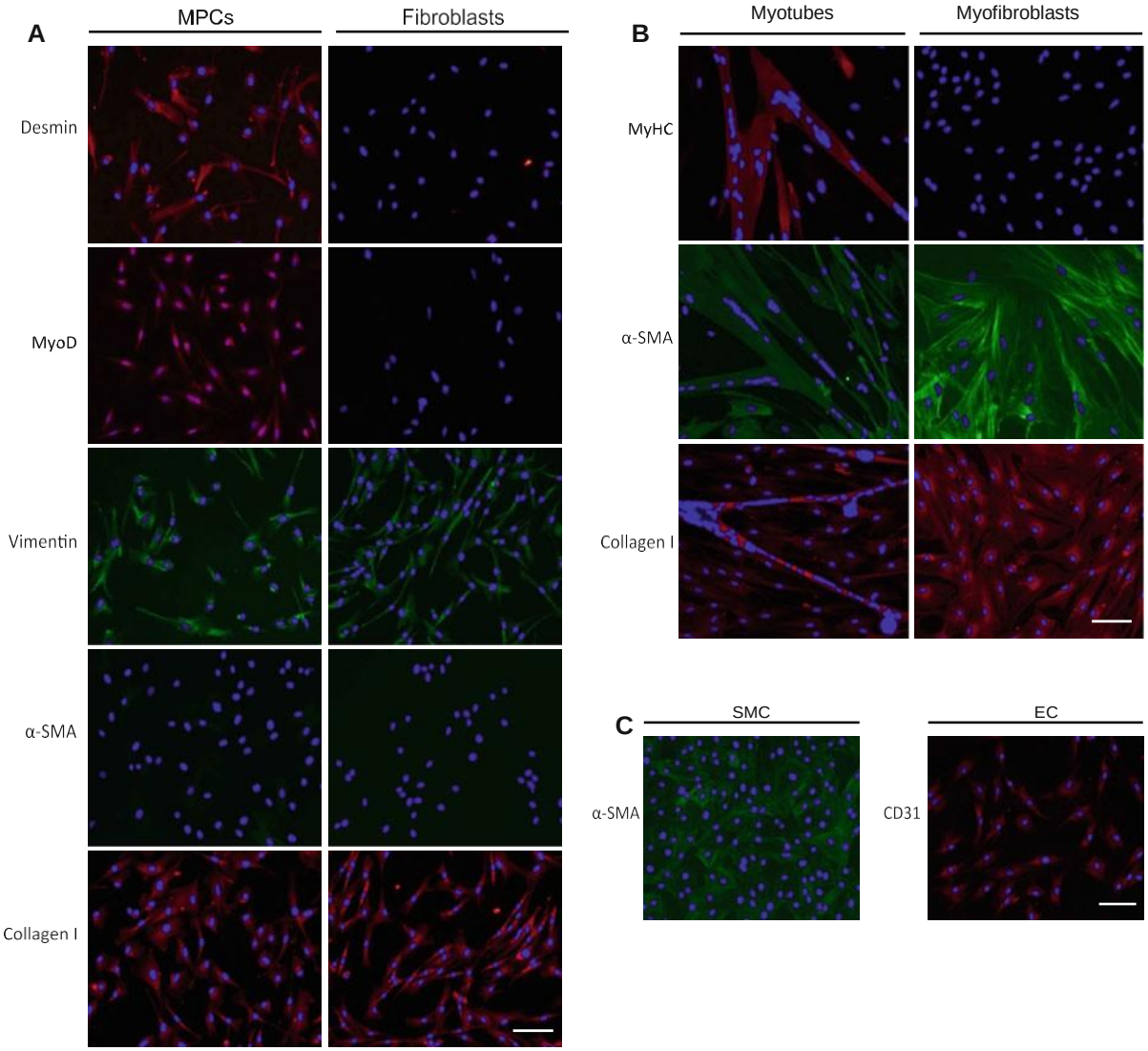

Fig. 2 Representative micrographs of immunofluorescent stains demonstrate high enrichment of separate cell isolations and culture populations of proliferating muscle precursor cells (MPCs) and muscle-derived fibroblasts (fibroblasts) individually, differentiated MPCs (myotubes) and differentiated fibroblasts (myofibroblasts), and smooth muscle cells (SMC) and endothelial cells (EC). All cell culture samples are primary cell isolations from young adult rat tissues. a Primary MPCs and fibroblasts, isolated from 3-mo-old skeletal muscle using the dual-isolation technique described in this study, were stained for myogenic markers desmin (*red*) and MyoD (*red*), as well as vimentin (*green*), *a*-smooth muscle actin (*a*-SMA) (*green*), and collagen I (*red*). Myogenic markers, desmin and MyoD, are highly expressed in MPCs, but not in fibroblasts (*top pair of panels*). The *bottom three pairs of panels* indicate that vimentin, *a*-SMA, and collagen I are expressed in both MPCs and fibroblasts. b MPCs and fibroblasts were differentiated to form myotubes and myofibroblasts, respectively, then stained for myosin heavy chain (MyHC; using MF20 antibody) (*red*), *a*-SMA (*green*), and collagen I (*red*) to demonstrate highly enriched myogenic lineage by myotubes and the formation of an extracellular matrix produced de novo by myofibroblasts. c Primary rat SMCs were stained for *a*-SMA (*green*) and primary rat EC's (*red*) were stained for CD31 for use as positive controls. None of our MPC- and fibroblast-derived isolations showed CD31 staining (data not shown). Desmin, MyoD, MyHC, CD31 and collagen I were stained using a red fluorophore, whereas vimentin and *a*-SMA were stained using a green fluorophore. All slides were co-stained with DAPI, making nuclei appear *blue* (MyoD? nuclei appear *reddish-purple* in color due to the co-staining of red and blue fluorophores). *Scale bar* 100 lm. Immunostaining procedures were repeated on 2–3 separate samples. (Color figure online)

types as controls to confirm the absence of smooth muscle cells and endothelial cells in our MPC and fibroblasts cultures. None of our MPC- and fibroblast-derived isolations were positive for CD31 staining (data not shown). The micrographs in Fig. 2c indicate positive control staining for *a*-SMA in smooth muscle cells and CD31 in endothelial cells. The novel findings here demonstrate our ability to isolate highly enriched individual populations of MPCs and fibroblasts from the same muscle tissue in 3- and 32-mo-old rats.

Gene expression in myofibroblasts

Myofibroblasts are the primary cells responsible for the secretion of ECM during tissue remodeling. Since old skeletal muscle displays greater fibrosis, compared to young, we sought to characterize the gene expression profiles of myofibroblasts from 3- and 32-mo-old skeletal muscle so the mRNA levels of selected ECM and inflammatory genes were measured in myofibroblasts after 4 days in low-serum conditions. Collagen IV*a*1 (COL4*a*1), collagen IV*a*2 (COL4*a*2), and laminin 2 [a.k.a. merosin] mRNAs were measured to analyze the de novo production of ECM genes in cultured myofibroblasts from 3- and 32-mo-old skeletal muscle; whereas mRNA levels of interleukin-6 (IL-6), tumor necrosis factor *a* (TNF*a*), and TGF-*b*1 were measured because these proteins have previously been shown to modulate satellite cell proliferation and differentiation. Additionally, we aimed to determine if myofibroblasts were a source of these factors that could negatively affect muscle regeneration with aging. Myofibroblasts from 32-mo-old skeletal muscle expressed approximately twice the level of ECM mRNAs, compared to 3-mo-old (COL4*a*1: $P \setminus 0.01$; COL4*a*2: $P \setminus 0.01$; Laminin 2: $P = 0.02$; Fig. 3a). Interestingly, levels of mRNA for IL-6 ($P = 0.01$), TNF*a* ($P \setminus 0.01$), and TGF-*b*1 ($P \setminus 0.01$) were all upregulated 2- to 4-fold in myofibroblasts from 32-mo-old skeletal muscle, relative to 3-mo-old (Fig. 3b).

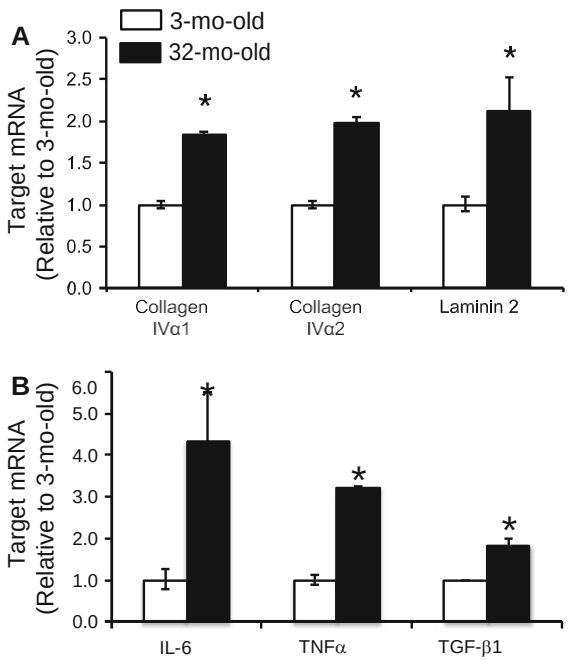

Fig. 3 qPCR results for mRNA expression of selected a extracellular matrix genes (collagen 4*a*1, collagen 4*a*2, and laminin 2), and b inflammatory genes (IL-6, TNF*a* and TGF-*b*1) in myofibroblasts (differentiated fibroblasts) cultured independently, but isolated from same skeletal muscle of 3-mo-old (*open bars*) or 32-mo-old (*filled bars*) rats using the dual-isolation techniques described in this study. Myofibroblasts from 32-mo-old skeletal muscle displayed increased levels of extracellular matrix mRNA (*2-fold higher than 3-mo-old) and inflammatory cytokine mRNA (2- to 4-fold higher than 3-mo-old). mRNA expression is normalized to 18S rRNA. $n = 4$ rats for both experiments for all groups. *Data* are represented as mean ± SEM. *Asterisk* denotes significant difference from 3-mo-old ($P \setminus 0.05$)

Skeletal muscle fibroblasts: BrdU incorporation and gene expression

The aberrant proliferation of fibroblasts in skeletal muscle tissue could potentially contribute the development of fibrosis during regeneration. To determine if there was a difference in proliferation between fibroblasts isolated from 3-mo-old and 32-mo-old skeletal muscle, BrdU incorporation was measured at 24 and 48 h after passaging. BrdU incorporation tended to increase ($P = 0.055$) in 32-mo-old fibroblasts at 24 h, compared to 3-mo-old (Fig. 4), with no significant difference in BrdU incorporation at 48 h. These data do not support fibroblast proliferation contributing to the development of fibrosis in old skeletal muscle under experimental considerations of same culture media; but do not exclude the possibility of differential proliferative responses from differences in proliferation factor activities between young and old muscle milieu.

Transforming growth factor-*b*1 (TGF-*b*1) and Delta/Notch signaling are important regulators of the regenerative process in skeletal muscle. To determine

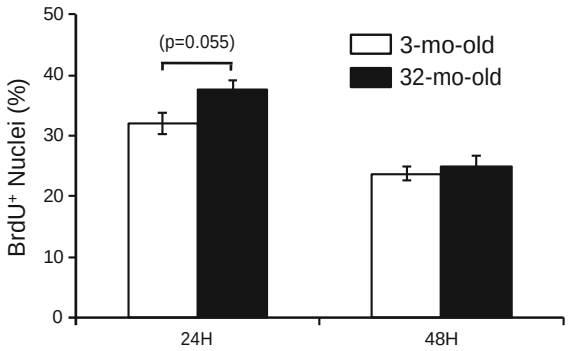

Fig. 4 BrdU incorporation was measured as an index of the state of proliferation at 24 and 48 h in passage one fibroblasts cultured independently, but isolated from 3-mo-old (*open bars*) or 32-mo-old (*filled bars*) skeletal muscle using the dual-isolation techniques described in this study. BrdU incorporation tended to be higher (17 %; $P = 0.055$) at 24 h in fibroblasts from 32-mo-old skeletal muscle, compared to 3-mo-old. $n = 4$ rats for both experiments for all groups. *Data* are represented as mean ± SEM

if gene expression was different between fibroblasts isolated from 3- and 32-mo-old skeletal muscle, levels of TGF-*b*1, Delta 1, Delta 3, and Delta 4 mRNAs were analyzed in proliferating MPCs at 24 and 48 h after passage. According to the Rat Genome Database (Medical College of Wisconsin; URL: http://rgd.mcw.edu/) there are three separate Delta ligand genes in the *Rattus norvegicus* genome: Delta 1 (Dll1), Delta 3 (Dll3), and Delta 4 (Dll4). It is unknown which Delta gene is most important in the skeletal muscle regenerative process and previous research in satellite cells from young and old skeletal muscle has not specified which Delta ligand was assessed (Carlson et al. 2008; Conboy et al. 2003); therefore, we investigated all three Delta ligand genes in this study. Delta 1 mRNA was significantly increased in 32-mo-old fibroblasts at 24 and 48 h ($P \setminus 0.01$ and $P = 0.03$, respectively), compared to 3-mo-old (Fig. 5b). No differences in mRNA levels were detected for TGF-*b*1 (Fig. 5a), Delta 3 (Fig. 5c), or Delta 4 (Fig. 5d), between 3- and 32-mo-old fibroblasts at either tested time point.

Muscle precursor cells: BrdU incorporation and gene expression

There is much debate as to whether satellite cells from old skeletal muscle possess an intrinsic impairment to proliferate, compared to young; therefore, we sought to investigate if a difference in proliferation exists in isolated and cultured MPCs. MPCs from 3- and 32-mo-old skeletal muscle were examined for BrdU incorporation at 24 and 48 h after passage. There was no significant difference in BrdU incorporation between 3-mo-old and 32-mo-old MPCs, at either tested time point (Fig. 6).

As with the proliferating fibroblasts experiments (Fig. 5), MPCs were analyzed for TGF-*b*1, Delta 1, Delta 3, and Delta 4 mRNAs. The rationale was that if there was a difference in gene expression between the ages, this could potentially contribute to the regenerative defect seen in aged muscle tissue. TGF-*b*1 mRNA was significantly 40–80 % higher in 32-mo-old MPCs at 24 and 48 h ($P = 0.05$ and $P = 0.04$, respectively), compared to 3-mo-old (Fig. 7a). Conversely, Delta 1 mRNA was significantly *65 % lower in 32-mo-old MPCs at both 24 and 48 h ($P \setminus 0.01$ and $P \setminus 0.01$, respectively), compared to 3-mo-old (Fig. 7b). Delta 3 mRNA was not different between ages (Fig. 7c) and Delta 4 mRNA was undetectable in MPCs (data not shown).

Discussion

The local, aged skeletal muscle environment is thought to contribute to satellite cell dysfunction (Gopinath and Rando 2008) and increased fibrosis (Kragstrup et al. 2011; Serrano et al. 2011), which, in turn, likely contributes to the progression of sarcopenia and impaired muscle regeneration and function (Brack et al. 2007). Due to the abundance of fibroblasts in skeletal muscle tissue, their close proximity to the satellite cell niche, and the role that they play in skeletal muscle regeneration, we sought to characterize the transcriptional profiles of certain genes in skeletal muscle fibroblasts, myofibroblasts derived from the isolated fibroblasts, and MPCs that all originated from the same muscle sample taken from 3- or 32-mo-old rats. To accomplish this, we established a primary cell isolation procedure by which MPCs and skeletal muscle fibroblasts are isolated from the same tissue sample from young and old skeletal muscles. Using this new procedure, we report herein the following novel findings. First, we identified a different transcriptional pattern in myofibroblasts from 32-mo-old skeletal muscle with increased levels of mRNA for the ECM components, collagen IV*a*1,

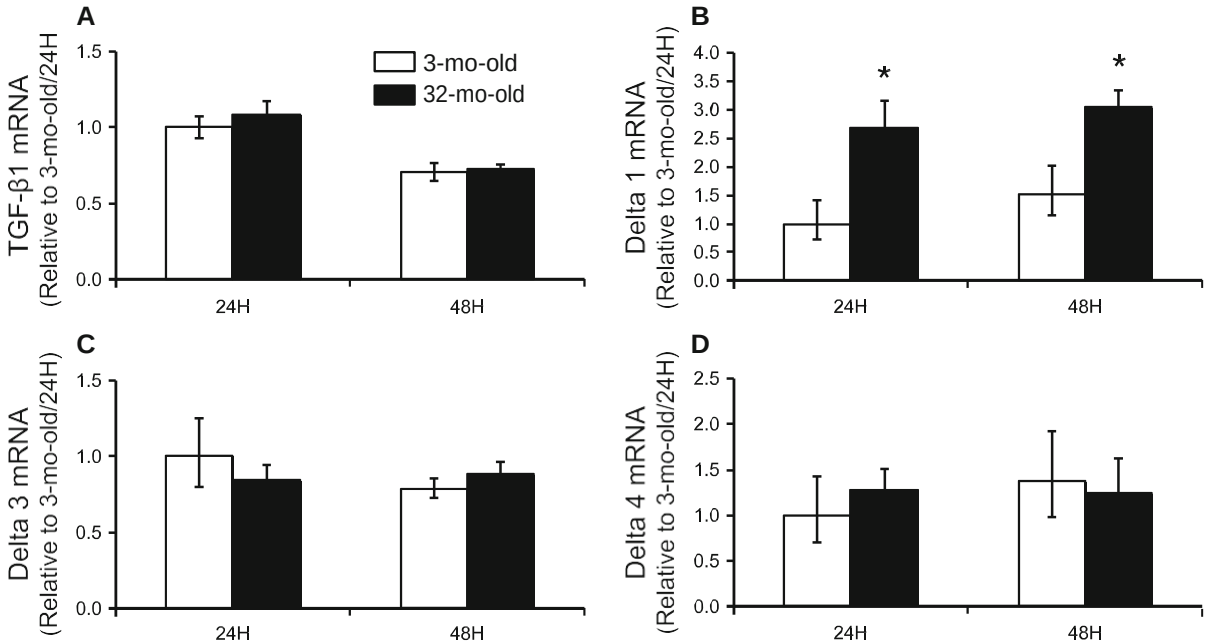

Fig. 5 qPCR results for mRNA expression of a TGF-*b*1, b Delta 1, c Delta 3, and d Delta 4 in proliferating fibroblasts isolated from skeletal muscle of 3-mo-old (*open bars*) or 32-mo-old (*filled bars*) rats at 24 or 48 h post-first passage. Fibroblasts were cultured independently, but isolated using the dual-isolation techniques described in this study. Delta-1 mRNA (b) was significantly higher ($P \setminus 0.05$) in fibroblasts from 32-mo-old skeletal muscle at both time points, compared to 3-mo-old. There was no difference in TGF-*b*1 (a), Delta 3 (c), or Delta 4 (d) mRNA levels between fibroblasts from 3- or 32-mo-old skeletal muscle. Expression is normalized to 18S rRNA. $n = 4$ rats for both experiments for all groups. *Data* are represented as mean ± SEM. *Asterisk* denotes significant difference from 3-mo-old

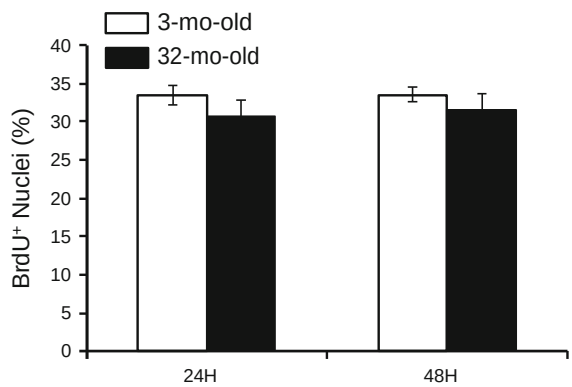

Fig. 6 BrdU incorporation was measured as an index of the state of proliferation at 24 and 48 h in passage one MPCs cultured independently, but isolated from 3-mo-old (*open bars*) or 32-mo-old (*filled bars*) skeletal muscle using the dual-isolation techniques described in this study. There were no significant differences in BrdU incorporation between 3-mo-old and 32-mo-old MPCs at either time point. $n = 8$ rats for both experiments for all groups. *Data* are represented as mean ± SEM

collagen IV*a*2, and laminin 2, as well as increased levels of mRNA for the inflammatory cytokines IL-6 and TNF*a*, and finally increased levels of TGF-*b*1 mRNA. Second, we report increased levels of Delta 1 mRNA in proliferating fibroblasts from 32-mo-old skeletal muscle, compared to 3-mo-old. Third, proliferating MPCs from 32-mo-old skeletal muscle dis- played increased levels of TGF-*b*1 mRNA, but decreased levels of Delta 1 mRNA. Most important, the different transcriptional patterns displayed between myofibroblasts, fibroblasts, and MPCs from 3-mo-old and 32-mo-old skeletal muscle were generated from identical culture conditions. One possible interpretation of these observations could be that cells from old skeletal muscle are "preprogrammed" with different transcriptional patterns that could alter the myogenic and fibrogenic properties of the muscle tissue with aging. Finally, we established and characterized a novel primary dual-cell isolation procedure, whereby MPCs and skeletal muscle fibroblasts are isolated from the same muscle tissue samples of 3- or

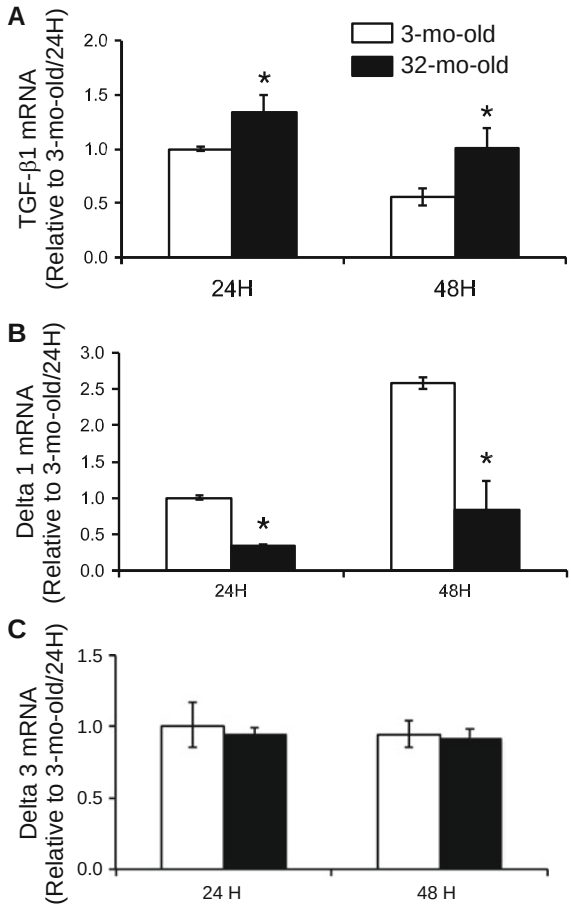

Fig. 7 qPCR results for mRNA expression of a TGF-*b*1, b Delta 1, and c Delta 3 in proliferating MPCs from skeletal muscle of 3-mo-old (*open bars*) or 32-mo-old (*filled bars*) rats at 24 or 48 h post-first passage. MPCs were cultured independently, but isolated using the dual-isolation techniques described in this study. MPCs from 32-mo-old skeletal muscle displayed increased levels of TGF-*b*1 mRNA (a; $P \setminus 0.05$ for both 24 and 48 h), but decreased levels of Delta 1 mRNA (b; $P \setminus 0.05$ for both 24 and 48 h). Delta 3 mRNA (c) levels were not different between 3- and 32-mo-old MPCs at either time point. Delta 4 mRNA was below the detectable limits in proliferating MPCs from 3- or 32-mo-old skeletal muscles (data not shown). Expression is normalized to 18S rRNA. $n = 4$ rats for both experiments for all groups. *Data* are represented as mean ± SEM. *Asterisk* denotes significant difference from 3-mo-old

32-mo-old rats. While we understand that the results presented in this study characterize myofibroblasts, fibroblasts, and MPCs from skeletal muscle of sedentary young and old rats, we believe that these findings have very important implications for the mechanisms involved in the process of skeletal muscle regeneration with aging.

Myofibroblasts from 32-mo-old skeletal muscle display different transcriptional patterns

Aged skeletal muscle is characterized by an increase in fibrous tissue, as muscle is replaced by connective tissue. Brack et al. (2007) showed that the aging-associated conversion of MPC to fibroblasts was mediated by systemic factors. Given the abundance of fibroblasts and ECM components in skeletal muscle tissue, their close proximity to satellite cells, and their potential influence on muscle regeneration, we hypothesized that myofibroblasts derived from fibroblasts would exhibit a different transcriptional pattern and might provide additional insights to contributors of dysfunctions in old skeletal muscle. Our novel observations thus extend the findings of Brack et al. (2007) in which they reported that MPCs isolated from 24-mo-old mice tended to have greater percentage of conversation from MPCs to fibrogenic lineage, as compared to 6-mo-old mice. In contrast to MPCs where the systemic environment of old mice mediated the MPC conversion to fibroblasts, we report for the first time that myofibroblasts differentiated from fibroblasts isolated from skeletal muscles of 32-mo-old rats displayed increased levels of mRNA for pro-inflammatory cytokines, as compared to myofibroblasts derived from 3-mo-old fibroblasts in identically composed culture media. We posit that the enhanced expression of pro-inflammatory genes of old myofibroblasts could likely contribute to chronically low inflammation milieu in old skeletal muscle (as discussed in the next paragraph).

Previous microarray studies from our laboratory demonstrated that old skeletal muscle tissue displays substantially different gene expression profiles, compared to young (Pattison et al. 2003a, b, c). In these reports, a majority of the differences in expression between 3- and 32-mo-old muscle were genes related to the ECM and the immune or stress responses. With that in mind, we sought to examine whether fibroblasts, isolated from 3- and 32-mo-old skeletal muscle, cultured, and then stimulated to differentiate into myofibroblasts would display differing mRNA expression profiles. Indeed, collagen IV*a*1, collagen IV*a*2, and laminin 2 (some key ECM molecules) were increased approximately twofold in myofibroblasts differentiated from fibroblasts of 32-mo-old muscle, compared to myofibroblasts generated from 3-mo-old fibroblasts. Further, the 32-mo-old myofibroblasts

expressed approximately threefold greater pro-inflammatory mRNAs for IL-6 and TNF*a*. One limitation of these findings is that it is unknown if levels of mRNA for these gene targets translate to differences in functional protein expressions in myofibroblasts from 32-mo-old skeletal muscle.

Investigations have reported that elevated plasma concentrations of IL-6 and TNF*a* in the elderly are associated with lower muscle mass and lower muscle strength in well-functioning older men and women (Visser et al. 2002; Schaap et al. 2009). The translational/clinical significance of increased IL-6 and TNF*a* mRNA levels in myofibroblasts may be that elevated levels of mRNA could elicit processes leading to increased IL-6 and TNF*a* secretions and an autocrine or paracrine effect in old skeletal muscles. The pro-inflammatory effects of IL-6 and TNF*a* have been shown to be concentration and time dependent. Thus it is possible that if inflammatory cytokines exceed a certain threshold and persist for a long enough time, they could have negative effects on aged skeletal muscle (Mathur and Pedersen 2008; Degens 2010). The twofold increase in TGF-*b*1 mRNA observed here in myofibroblasts from 32-mo-old muscle is consistent with the finding that TGF-*b*1 appears to co-localize around the basement membrane of old myofibers, but not young (Carlson et al. 2008). It is possible that myofibroblasts play a role, together with MPCs, in the increased production of TGF-*b*1 observed in aged skeletal muscle tissue, thus contributing to the aged skeletal muscle phenotype. Taken together, these results support the hypothesis that the aged skeletal muscle tissue environment, particularly the role of the fibroblasts/myofibroblasts, may contribute to the increased fibrosis and impaired regenerative capacity of skeletal muscle with aging.

TGF-*b*1 and Delta in proliferating fibroblasts and MPCs

Delta 1 mRNA was almost threefold higher in fibroblasts isolated from 32-mo-old skeletal muscle, compared to 3-mo-old; however, no changes in mRNAs for Delta 3, Delta 4 or TGF-*b*1 were noted. Delta protein is a membrane-bound ligand that interacts with the Notch receptor on a second cell (Six et al. 2004). Signaling through Notch-like receptors is an evolutionarily well-conserved mechanism for cell–cell communication (Lendahl 1998) to determine cell fate specification (Six et al. 2004). Conserved Notch ligands include the Serrate orthologs Jagged-1 and -2 and the Delta orthologs Delta-1, -3, and -4 (Six et al. 2004). Intriguingly, Delta ligand-Notch receptor interactions have been shown to play some role in fibroblast specification to myofibroblasts. The Notch receptor ligand family member Jagged-1 application to healthy dermal fibroblasts resulted in differentiation of resting fibroblasts into myofibroblasts and release of collagen (Dees et al. 2011). Furthermore, activation of the Notch signaling pathway has been observed for FIZZ1 induction of Jagged 1 to cause myofibroblast differentiation in lung fibroblasts (Liu et al. 2009). Together, future studies should further examine the role of Delta 1 as a potential factor stimulating the differentiation of skeletal muscle fibroblasts to myofibroblasts.

On another note, levels of TGF-*b*1 mRNA, rather than Delta 1 mRNA, were increased in MPCs from 32-mo-old skeletal muscle, compared to 3-mo-old. Conboy et al. (2003) demonstrated that injury to skeletal muscle in 24-mo-old mice caused a greater increase of TGF-*b*1 protein than in 2-mo-old; which we suggest could be interpreted as in agreement with our findings, due to the process of isolation and culture initiating the regenerative process of MPC proliferation. Further, Conboy et al. (2003) observed an insufficient up-regulation of the Delta protein in regenerating skeletal muscle of older 24-mo-old mice. Together, the 50 % lower expression of Delta 1 mRNA in MPCs from 32-mo-old skeletal muscle, along with no change in Delta 3 mRNA and no detection of Delta 4 mRNA observed in the current study, we posit that impaired Delta 1 mRNA expression may be the responsible for insufficient up-regulation of the Delta protein in injured muscle of older mice as previously reported (Conboy et al. 2003). Unlike previous reports demonstrating that age-associated differences in Delta (Conboy et al. 2003) and TGF-*b*1 (Carlson et al. 2008) expression are likely responsible for the differences observed in satellite cell proliferation with aging, increased expression of TGF-*b*1 mRNA and decreased expression of Delta 1 mRNA in 32-mo-old MPCs, from the current study, did not correspond with differences in BrdU incorporation between 3- and 32-mo-old MPCs. This discrepancy may be attributed to differences in cell culture conditions (e.g. number of cell passages and timing of assessing

proliferation) or age and species of animal used for experimentation.

Novel model for the isolation and culture of MPCs and fibroblasts

Simultaneous isolation of MPCs and fibroblasts from same sample of skeletal muscle minimizes variation between two separate muscle samples. Further, extension of the protocol to allow conversion of fibroblasts to myofibroblasts allowed three cell types to be examined from the same muscle sample. Immunohistochemistry demonstrated that MPC and fibroblast cell populations were highly enriched, even in one-passage generations. To further validate and characterize our skeletal muscle fibroblast isolation and culture techniques, we differentiated primary fibroblasts (myofibroblasts) to determine if they would produce ECM proteins. Indeed, myofibroblasts were positive for *a*-SMA and collagen I, but negative for MyHC protein, confirming absence of MPCs and that myofibroblasts were derived from primary fibroblast cultures as they synthesized ECM proteins.

In an attempt to further develop our cell culture model towards a model that more closely represents skeletal muscle tissue in vivo, we co-cultured MPCs with myofibroblasts isolated from 3-mo-old skeletal muscle (Supplemental Fig. 1). This novel preliminary work suggests that this may be a feasible culture model for primary muscle tissue culture that allows for the characterization of fibroblast–myoblast interactions; however, further studies are needed to examine the effects of co-culturing MPCs and myofibroblasts from young and old skeletal muscle. In the future, these techniques may be useful in future research that investigates the roles that both myogenic and non-myogenic cell types play in skeletal muscle regeneration with aging, as well as demonstrating feasibility for studies limited by smaller skeletal muscle tissue samples, such as from human muscle biopsies.

Conclusion

In the present paper, we have established a novel model for the simultaneous sequential isolation of primary MPCs and fibroblasts from the same skeletal muscle tissue sample from 3- and 32-mo old rats, and in doing so, we introduced a co-culture model that may prove useful for future studies investigating satellite cell-fibroblast interactions. Then, we individually cultured myofibroblasts and proliferating MPCs and fibroblasts and reported the existence of novel differences in transcriptional patterns between cells from 3- and 32-mo-old skeletal muscle, characterized by the increased levels of mRNA for certain ECM and regulatory genes. Taken together, these findings support the notion that myofibroblasts and fibroblasts may play an important role in the regeneration potential of skeletal muscle and could have further implications into the mechanisms that contribute to the increased fibrosis observed in aged skeletal muscle. We believe these novel findings complement previous work and will advance future research investigating the mechanisms responsible for the impaired regeneration of aged skeletal muscle.

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