DOI: 10.1038/ncb2015

METHODS

Animals. All mice were maintained in a pathogen-free facility, and all experiments were performed in accordance with the University of British Columbia Animal Care Committee regulations. Adult C57BL/6 mice (> 8 weeks) were used in this study. C57BL/6-CMV-β actin-EGFP transgenic mice were a gift from I. Weissman (Stanford University, CA, USA). *In vivo* lineage tracing was performed in *Myf5–Cre–R26R3–YFP* mice, generated by breeding heterozygous *Myf5–Cre* (P. Soriano, Department of Developmental and Regenerative Biology, Mount Sinai School of Medicine, NY, USA) with *ROSA26–YFP*[44] reporter mice. Muscle damage was induced by intramuscular injection of 0.15 μg notexin snake venom (Latoxan), into the tibialis anterior muscle.

Tissue preparation. Skeletal muscle from both hindlimbs was carefully dissected and then gently torn with tissue forceps until homogeneous. Collagenase type 2 (Sigma; 2ml of 2.5 U ml^{-1}), in 10 mM $CaCl_2$, was added to every two hindlimbs, and the preparation was placed at 37 C for 30 min. After washing, a second enzymatic digestion was performed with Collagenase D (Roche Biochemicals; 1.5 U ml^{-1}) and Dispase II (Roche Biochemicals; 2.4 U ml^{-1}), in a total volume of 1 ml per mouse, at 37 C for 60 min. Preparations were passed through a 40-μm cell strainer (Becton Dickenson), and washed. Resulting single cells were collected by centrifugation at 400*g* for 5 min.

Flow cytometry/FACS. Cell preparations were incubated with primary antibodies for 30 min at 4 °C in supplemented PBS containing 2 mM EDTA and 2% FBS at ~3×10^7 cells per ml. We used the following monoclonal primary antibodies: anti-CD31 (clones MEC13.3, Becton Dickenson, and 390, Cedarlane Laboratories), anti-CD34 (clone RAM34, eBioscience), anti-CD45 (clone 30-F11, Becton Dickenson), anti-CD45.1 (clone A20, Becton Dickenson), anti-CD45.2 (clone 104, eBiosciences), anti-Sca-1 (clone D7, eBiosciences) and anti-α7 integrin (produced in-house). Typical antibody dilutions used were: anti-CD31, 1:100–400; anti-CD34, 1:50–200; anti-CD45, 1:200–400; anti-CD45.1, 1:200–400; anti-CD45.2, 1:200–400; anti-Sca-1, 1:2,000–5,000; anti-α7 integrin, 1:100–400. For all antibodies we performed fluorescence minus one controls by staining with appropriate isotype antibodies. Where necessary, biotinylated primary antibodies were detected using streptavidin coupled to phycoerythrin, allophycocyanin or FITC (Caltag). To assess viability, cells were stained with propidium iodide (1 μg ml^{-1}) and Hoechst 33342 (2.5 μg ml^{-1}) and resuspended at ~1×10^7 cells ml^{-1} immediately before sorting or analysis.

Analysis was performed on LSRII (Becton Dickenson) equipped with three lasers. Data were collected using FacsDIVA software. Sorts were performed on a FACS Vantage SE (Becton Dickenson) or FACS Aria (Becton Dickenson), both equipped with three lasers, using a 100-μm nozzle at 18 psi to minimize the effects of pressure on the cells. Sorting gates were strictly defined based on isotype control (fluorescence minus one) stains. Biexponential analysis was performed using FlowJo 8.7 (Treestar) software.

Cell culture. Cells were grown in high-glucose Dulbecco's modified eagle medium (DMEM), supplemented with 2.5 ng ml^{-1} bFGF (Invitrogen) 20% FBS and 10% heat-inactivated horse serum. This medium is referred to in text as 'growth medium'. Cells were seeded in tissue-culture-treated plates coated with Matrigel (Becton Dickenson) or Type 1 collagen (Sigma). After sorting, cells were allowed to adhere for three days, after which half the medium was changed. Media was changed every 2–4 days thereafter. For mesenchymal differentiation, we used reported conditions. Briefly, we cultured cells in DMEM with 20% FBS under the following conditions: for osteogenic differentiation, we supplemented media with 10 nM dexamethasone, 5 mM β-glycerophosphate and 50 μg ml^{-1} ascorbic acid; for adipogenic differentiation, we supplemented media with 0.25 μM dexamethasone and 0.5 mM isobutylmethylxanthine, 1 μg ml^{-1} insulin and 5 μM troglitazone; for chondrogenic differentiation we pelleted cells by centrifugation at 400*g* for 5 min, and grew them in media supplemented with 1 ng ml^{-1} TGFβ1 and 50 μg ml^{-1} ascorbic acid. For myogenic differentiation, we used DMEM medium supplemented with 5% horse serum.

Limiting dilution analysis. From each test population, 1–100 cells were sorted into individual wells of a Matrigel-coated 96-well plate directly from the sorter. Cells were grown as described in the text. After three weeks, cultures were fixed and stained for MyHC and nuclei. Wells were scored for the presence of colonies (> 8 cells), cells undergoing terminal myogenic differentiation (MyHC-positive) and lipid laden adipocytes. A minimum of 30 replicate wells was generated for each cell dose. Limiting dilution analysis calculations were based on the single hit Poisson model (see Statistics).

Gene expression analysis. RNA isolation was performed using RNeasy mini kits (Qiagen), and RNA quantification was performed using a ND1000 spectrophotometer (Nanodrop). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression analysis was performed using Taqman Gene Expression Assays (Applied Biosystems), on a 7900HT Real Time PCR system (Applied Biosystems). Sequence information for the primers contained in the Taqman assays are not in the public domain, but ordering information is provided in Supplementary Information, Table S3. Data were acquired and analysed using SDS 2.0 and SDS RQ Manager software (Applied Biosystems).

Transplantation. Host mice (wild type) were anesthetized using isoflurane. Donor cells were isolated from transgenic mice ubiquitously expressing GFP^+. Cells were sorted into cold DMEM and collected by centrifugation at 450*g* for 5 min. For subcutaneous transplantation, cells were resuspended in 25 μl Matrigel and loaded into an ice-cold needle and syringe immediately before injection. Cells were injected into the subscapular region, with control cells injected on the contralateral side. For intramuscular transplantation, sorted cells were resuspended in 20 μl sterile PBS and injected into the tibialis anterior. Tissues were collected after three weeks.

Histology and imaging. Before tissue collection, animals were perfused transcardially with 20 ml PBS/ 2 mM EDTA, followed by 20 ml 4% paraformaldehyde (PFA). Tissues were processed for cryosectioning or paraffin-embedding using standard methods. Immunostaining was performed using monoclonal antibodies against perilipin (Sigma), α smooth muscle actin (Sigma), ER-TR7 (Novus), FSP1 (Novus) myosin heavy chain (in-house) or BrdU (Becton Dickenson). Briefly, samples were permeabilized in 0.3% Triton X-100 (Sigma) in PBS, and blocked for 2 h at room temperature in PBS containing 25% normal goat serum (NGS), 0.3% Triton X-100, 3% bovine serum albumin (BSA) and 0.1% NaN_3. Cells were stained overnight at 4 °C using antibody diluted in 10% NGS, 0.3% Triton X-100, 3% BSA and 0.1% NaN_3. Primary antibody was detected using goat anti-mouse IgG antibodies conjugated to Alexa 568 or 488 (Molecular Probes), or using the anti-Mouse IgG HRP detection kit (Becton Dickenson).

Cells were visualized using a microscope (Axiovert 200 for inverted microscopy, Axioplan2 for conventional microscopy; Carl Zeiss Microimaging) and images were acquired using a charge-coupled device camera (Retiga Exi, Axiovert 200 or Retiga Ex, Axioplan2; qImaging) and OpenLab4 software (Improvision). To confirm the specificity of the GFP signal, we compared the signal with non-specific autofluorescent background in all other channels[45]. Confocal microscopy was performed using a Nikon C1 laser scanning confocal microscope equipped with lasers at 488 nm, 568 nm and 633 nm. Images were captured using the least exposure time possible, and manipulation of brightness and contrast, colouring adjustments and assembly into figures were performed using ImageJ, OpenLab4 (Improvision), Illustrator CS3 (Adobe) and Photoshop CS3 (Adobe).

BrdU-labelling studies. For *in vivo* studies, BrdU was administered in drinking water (0.8 g l^{-1} in 2% sucrose) and by intraperitoneal injection (100 mg kg^{-1}). In cell culture experiments, 10 μM BrdU was added to culture medium. BrdU treatment regimens are described in the text. For flow cytometric analysis, cells were stained for surface markers as indicated. Samples were fixed in 2% PFA for 20 min, and membranes were permeablized in 0.2% saponin for 10 min. Cells were treated with 30 μg DNAse I (Sigma) for 1 h at 37 °C, after which a 1:25 dilution of anti-BrdU (clone PRB-1, Invitrogen) was applied to the samples for 30 min at room temperature.

MTT assay. Assays were performed in 96-well cell cultures. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (50 μl of 5 mg ml^{-1} dissolved in PBS) was added to each well and samples were incubated at 37 °C for 4 h. The solution was removed, and the purple formazan salt product resulting from the reduction of the yellow MTT was solublized in 100 μl DMSO and quantified spectrophotometrically at 570 nm (SpectraMax 190, Molecular Devices).

Statistics. Preliminary analysis and data collation was performed using Microsoft Excel. Statistical tests, including Student's *t*-test, ANOVA and regression analyses were performed using Prism 4 (GraphPad Software). Analysis of limiting dilution data were performed using a web application made available by the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia (http://bioinf.wehi.edu.au/software/limdil/)[46]. This software tests departures from the single-hit Poisson model using a generalized linear model. Error bars in all figures, including Supplementary Information, represent the mean ± s.e.m., with the exception of Fig. 7a, which represent the mean ± s.d. Raw data points for all experiments presented as averages in the main figures are available in Supplementary Information, Table S2.

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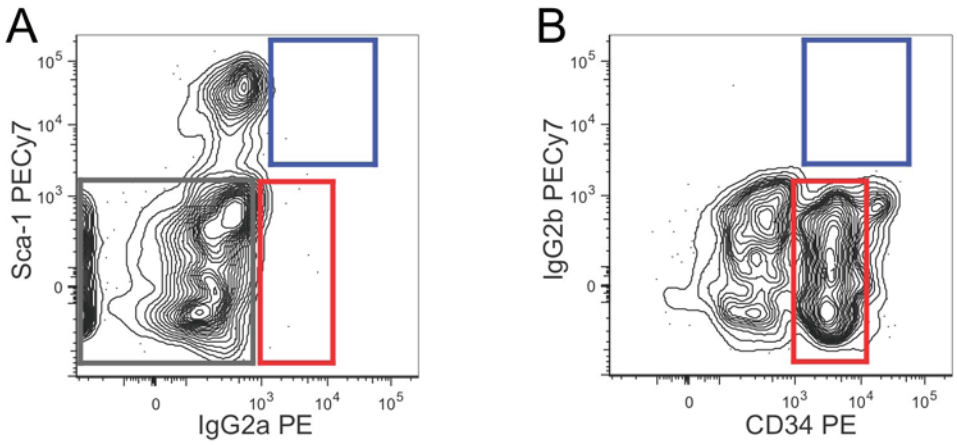

Figure S1 Confirmation of Sca-1 CD34 stain specificity using isotype-matched control antibodies. Skeletal muscle preparations were stained and gated as described in the text (Hoechst^{mid} PI^{lo} CD45^{-} CD31^{-}). Isotype stains were the same as normal stains except that one antibody was substituted with an isotype-matched, non-specific antibody, conjugated to the same fluorophore. (**A**) CD34 PE Isotype control. (**B**) Sca-1 PECy7 Isotype control.

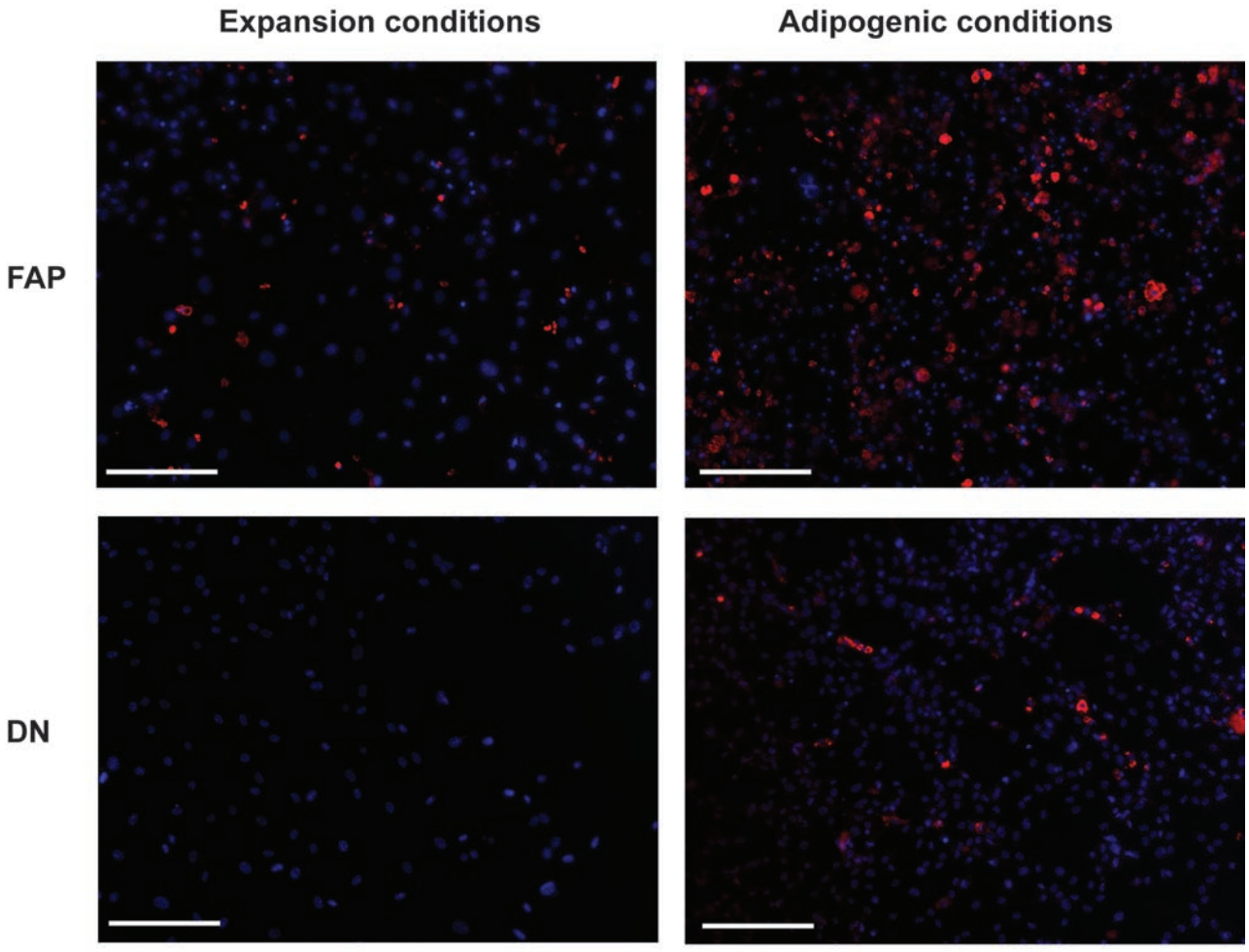

Figure S2 A latent adipogenic potential of skeletal muscle Sca-1$^-$ CD34$^-$ (DN) cells is revealed in the presence of PPARγ agonists. DN cells and FAPs were FACS purified as described and cultured in either expansion conditions or adipogenic conditions containing PPARγ agonists for 17 days. Samples were stained with myosin heavy chain and perilipin to identify differentiated cells. Independently of the conditions, neither cell type gave rise to MHC positive structures, suggesting that these two cell subsets do not possess myogenic potential. FAPs spontaneously gave rise to perilipin positive cells in expansion conditions and, more efficiently, in adipogenic conditions. DN cells were capable to generate small numbers of perilipin expressing adipocytes only when exposed to adipogenic medium. Scale bar: 100μm

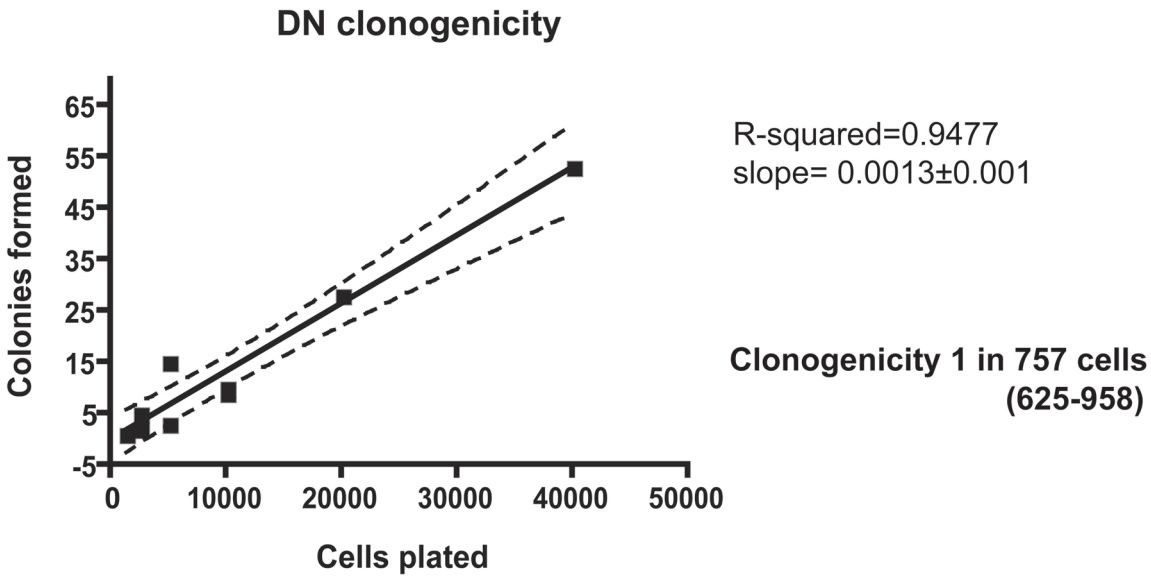

Figure S3 Linear regression of colony forming data from freshly isolated skeletal muscle DN (Sca-1$^-$ CD34$^-$) cells. Hoechst^{mid} PI^{lo} CD45$^-$ CD31$^-$. Sca-1$^-$ CD34$^-$ cells were sorted and seeded at the indicated densities in a 5cm matrigel-coated dish and cultivated in growth medium for 2 weeks. After fixation, colonies (defined as clusters of more than 10 cells) were counted, and then plotted against the number of cells inoculated. Dashed lines represent the 95% confidence regression band. Clonogenicity was determined from the slope of the curve, and the 95% confidence interval is shown in brackets.

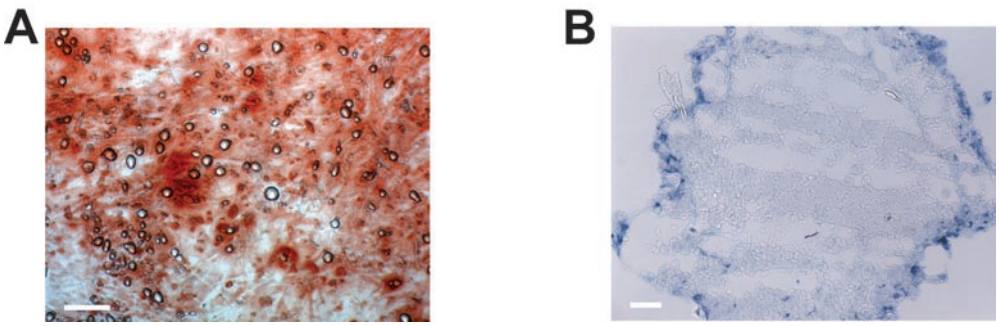

Figure S4 Skeletal muscle Sca-1^{+} CD34^{+} cells do not form bone or cartilage. Cells were previously gated for Hoechst^{mid} PI^{lo} CD45^{-} CD31^{-}. (**A**) DP cells were grown in osteogenic conditions for 10 weeks, and then stained with alizarin red for visualization of calcified nodules. Despite the presence of alizarin red staining, we observed no areas that could be identified as osteogenic nodules. Sparse adipocytes were observed throughout the cultures. Scale bar at 50μm. (**B**) DP cell pellets were grown in chondrogenic conditions for 8 weeks, cryosectioned (10μm), and then stained for glycosaminoglycans with alcian blue. Although non-specific staining could be observed on the periphery of the pellet, no staining was observed throughout the pellet, and no characteristic chondrocyte morphology was observed. (Scale bar at 50μm).

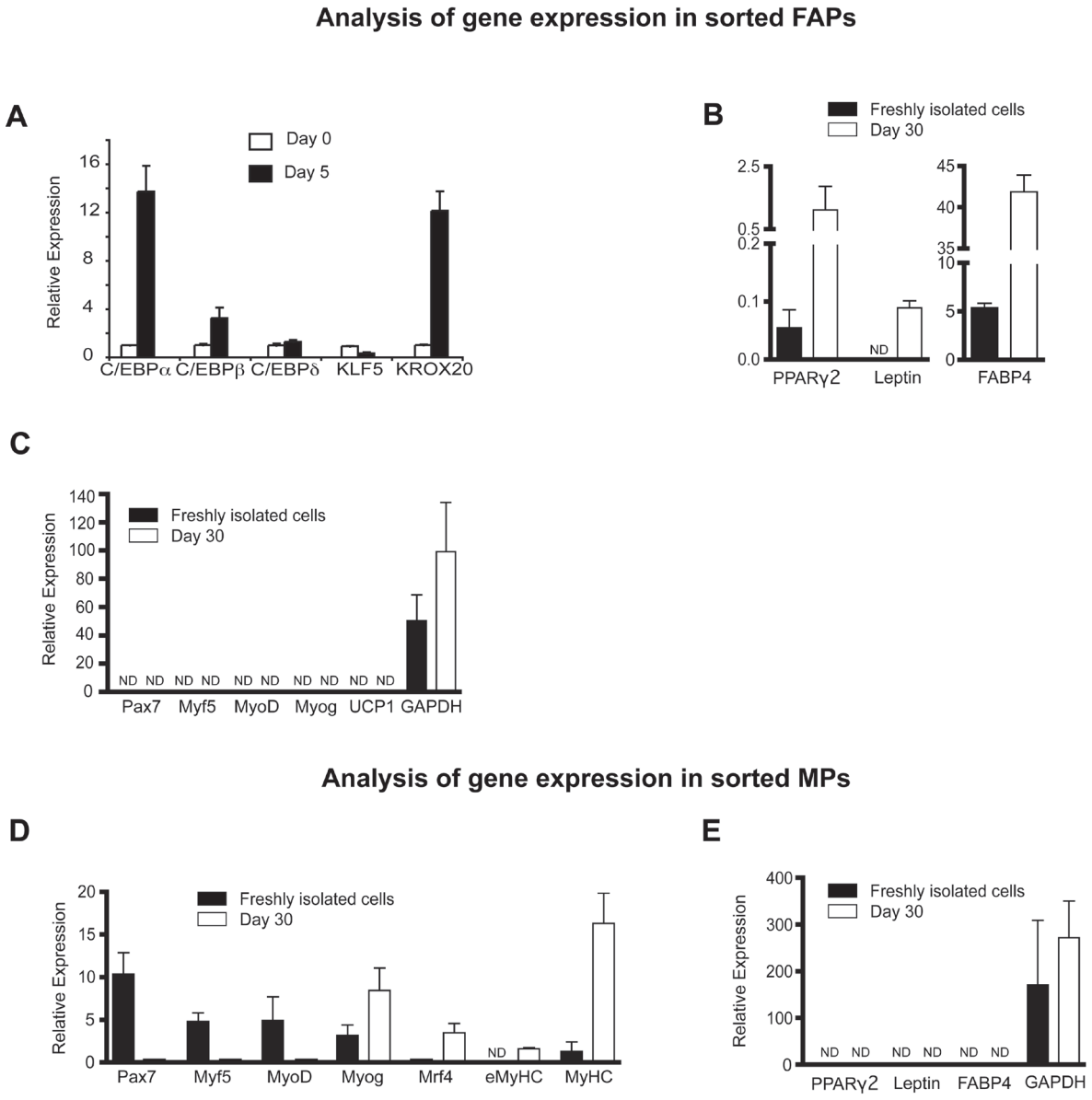

Figure S5 Quantitative gene expression in sorted cells. Muscle-resident adipogenic (lin$^-$ Sca-1$^+$ CD34$^+$) and myogenic (lin$^-$ Sca-1$^-$ CD34$^+$) cells were sorted and cultured as described in text. Differentiated cells arose spontaneously in growth media and were analyzed at the time points indicated. We performed qRT-PCR analyses using Taqman probe and primer sets spanning exon-exon boundaries. All values are expressed relative to TBP expression. ND= no signal detected. (**A**) Upregulation of preadipocyte markers on FAPs after 5 days of culture. (**B**) Upregulation of markers of mature adipocytes in FAPs after terminal differentiation (30 days). (**C**) Adipogenic cells do not express skeletal muscle or brown fat genes. (**D**) Myogenic cells express markers common to satellite cells immediately after sorting, and markers of mature and regenerating muscle after terminal differentiation. (**E**) Myogenic cells do not express genes common to white adipocytes.

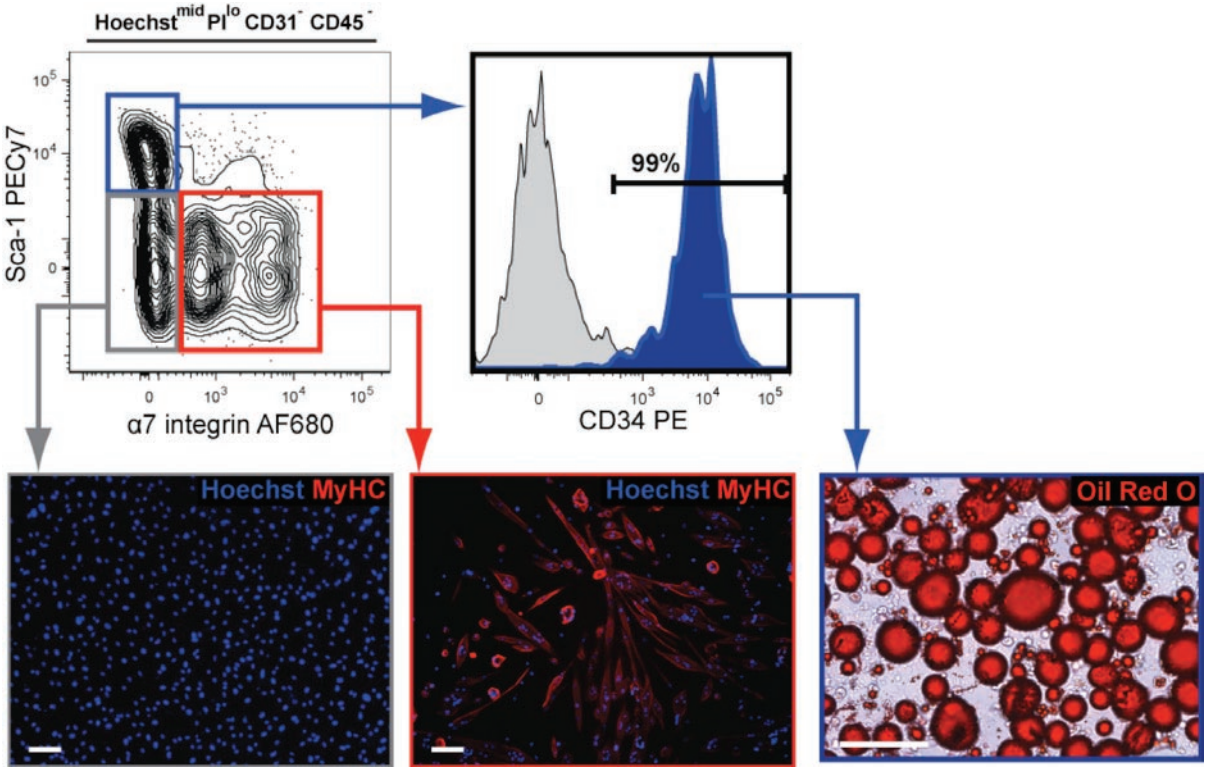

Figure S6 An alternative strategy using α7 integrin to identify adipogenic and myogenic cells from dissociated skeletal muscle. Skeletal muscle preparations were gated for Hoechst^{mid} PI^{lo} CD45$^-$ CD31$^-$ (lin$^-$) cells, and then analyzed for the expression of Sca-1 and α7 integrin. Lin$^-$ Sca-1$^-$ α7$^-$ cells did not differentiate into myosin heavy chain (MyHC)-expressing myotubes (grey gate, left image), revealing that all myogenic activity was found exclusively in the lin$^-$ α7$^+$ Sca-1$^-$ population (red gate, middle image). All adipogenic activity was found exclusively in the lin$^-$ α7$^-$ Sca-1$^+$ population (blue gate, right image), and over 99% of these cells expressed CD34. Greyed out histogram represents the CD34-PE isotype control. All scale bars at 100μm.

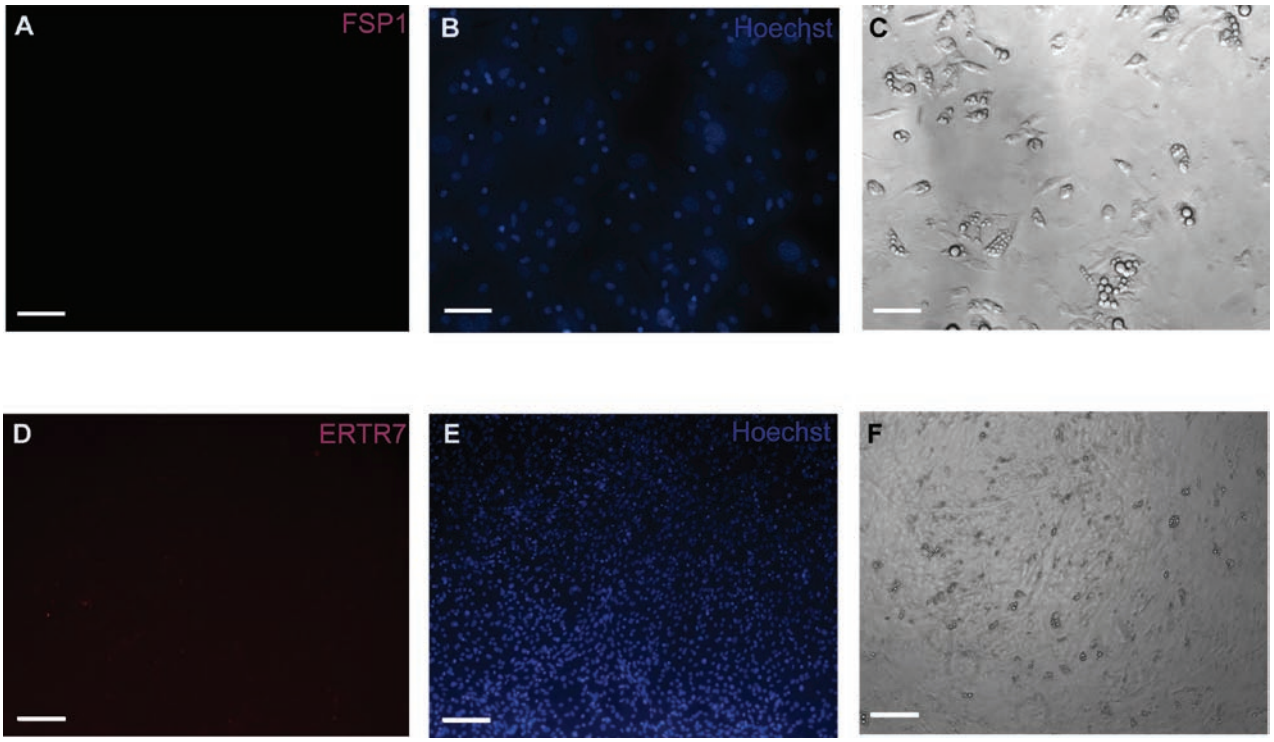

Figure S7 Negative controls for ER-TR7 and FSP-1 staining of FAPs. No staining was detected when parallel cultures of FAPs were stained in an identical fashion as those shown in Fig 2, but omitting the primary antisera. (**A**, **B**, **C**) FSP1 controls. Scale bars: 50μm B) shows Hoechst staining and (**C**) brightfield image of the sample shown in (**A**. **D**, **E**, **F**) ER-TR7 controls. Scale bars: 100μm (**E**) shows Hoechst staining and (**D**) brightfield image of the sample shown in **D**.

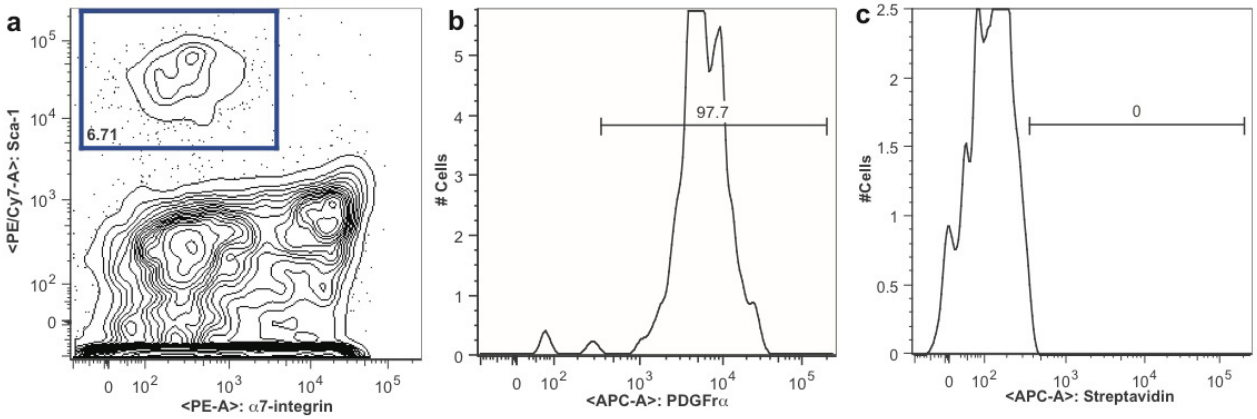

Figure S8 Expression of PDGFRα in CD31$^-$, CD45$^-$, Sca1$^+$, α7-integrin$^-$ FAPs. The frequency of FAPs expressing PDGFRα was investigated by flow cytometry. Hoechst$^+$, PI$^-$, CD31$^-$, CD45$^-$ cells were gated for the expression of Sca-1 (**a**). Nearly all of these cells express PDGFRα, as shown in (**b**). (**c**) Negative control showing similarly stained and gated cells that were not exposed to the anti-PDGFRα antibodies.

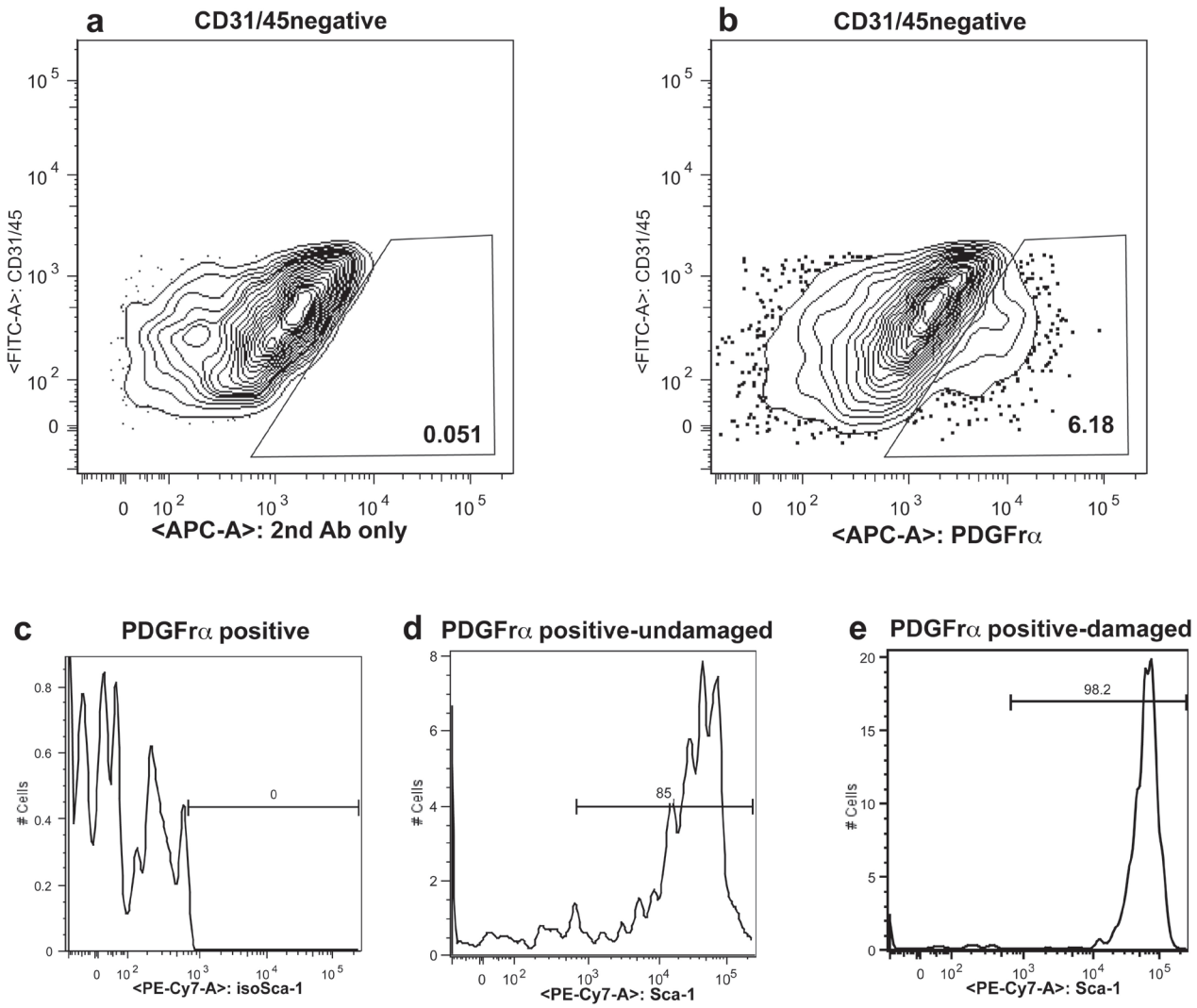

Figure S9 Sca-1$^+$, α7 integrin$^-$ cells are the main population expressing PDGFRα in skeletal muscle. The overlap between PDGFRα expressing cells and Sca1$^+$, α7 integrin$^-$ fibro/adipogenic progenitors was investigated in the CD31, CD45 negative fraction form dissociated muscle samples. (a) Negative controls that were not incubated with anti-PDGFR-biotin primary antibody confirm the specificity of the stain and were used to set the gate identifying positive cells (shown in b). (c,d) PDGFRα$^+$ cells were stained with either anti-Sca-1 antibodies or with isotype-matched negative controls. (d) Over 85% of PDGFRα expressing cells also express Sca-1 (e) The percentage of PDGFRα expressing cells also expressing Sca-1 increased three day after damage . No α7-integrin expression was detected on PDGFRα positive cells (not shown).

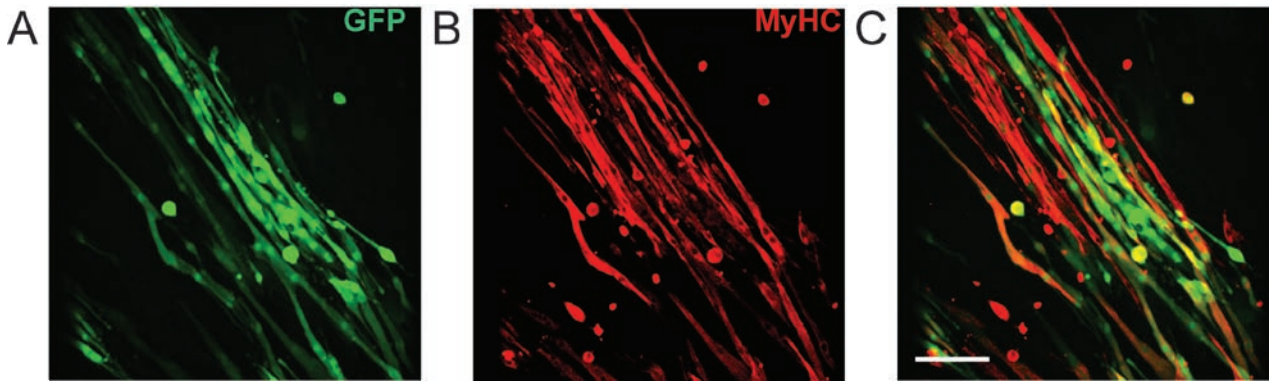

Figure S10 Co-cultivation control experiment. This figure shows data from a control experiment for Figure 3A. Equal numbers of MPs sorted from transgenic GFP$^+$ and wildtype animals were co-cultivated for 14 days. Participation of both GFP$^+$ and GFP$^-$ MPs to the myogenic lineage was confirmed by microscopy. Scale bar at 100μm. (**A**) GFP+ myotubes. (**B**) Myosin Heavy Chain immunostain. (**C**) Merge.

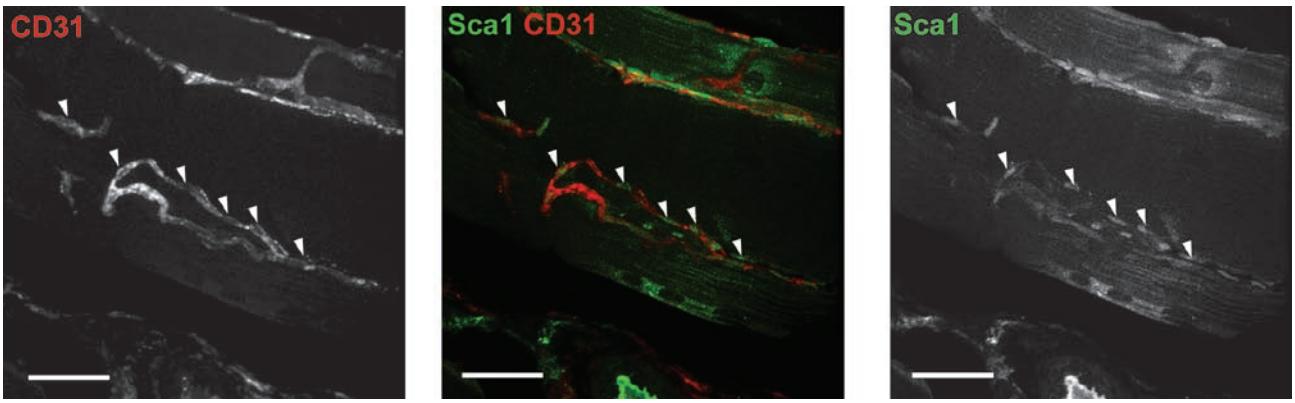

Figure S11 Sca-1$^+$ mononuclear cells appear associated with vessels in undamaged skeletal muscle. Sca-1$^+$ mononuclear cells are found in close contact with myofiber associated blood vessels in undamaged muscle. A maximum intensity projection of a confocal Z-stack is shown. Scale bar: 100μm.

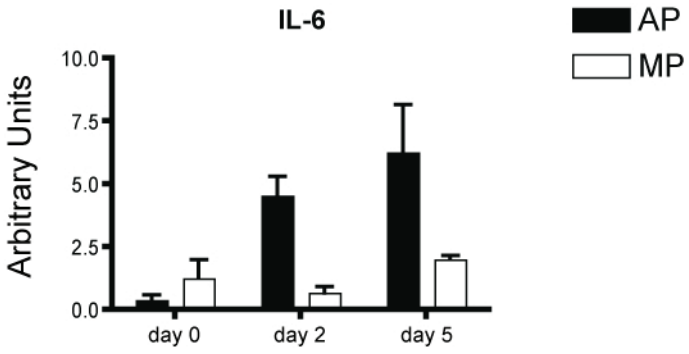

Figure S12 IL-6 expression is upregulated in FAPs in response to muscle damage. Following muscle damage, MPs and FAPs were sorted at the indicated time points and expression of IL-6 analyzed by qRT-PCR.

Supplementary Table 1. Summary table of the developmental potentials detected in the different culture conditions used.

DN cells and FAPs were FACS purified as described and cultured in either growth, osteogenic, chondrogenic or adipogenic conditions containing PPARγ agonists. Cultures were considered positive for bone based on the presence of mineralized (alizarin red positive) bone nodules. Positive for cartilage based on the presence of Alcian blue positive matrix. Positive for muscle based on the presence of myosin heavy chain positive structures. Positive for fat based on the presence of cells containing perilipin or oil red positive vacuoles.

Growth medium

	$CD34^-$ $Sca1^-$(DN)	$CD34^+$ $Sca1^-$ (MP)	$CD34^+$ $Sca1^+$ (FAP)
Bone	-	-	-
Muscle	-	+	-
Fat	-	-	+

Osteogenic medium

Bone	+	-	-
Muscle	-	+	-
Fat	-	-	+

Chondrogenic medium

Cartilage	+	-	-

Adipogenic medium

Muscle	-	+	-
Fat	+	-	+

Myogenic medium

Muscle	-	+	-

Supplementary table 2: Raw data for all experiments incuded

Figure 5c	(each data point comes from an individual animal)						
% BrdU+ APs							
BrdU Pulse Day							
0-1	0.00	1.32	0.59	0.38	0.08		
1-2	12.90	16.70	23.93	24.95	18.89		
2-3	25.00	17.10	8.94	40.08	21.17		
3-4	10.80	13.20	24.40	49.94	45.30	59.18	49.5
4-5	0.00	24.14	38.30	13.50	15.20	21.10	
5-6	3.45	5.13	9.73				
6-7	0.00	2.44	0.00				
9-10	0.00	0.00	0.00	1.07	3.39	1.29	
13-14	0.00	0.00	0.79	0.00	5.66		
% BrdU+ MPs							
BrdU Pulse Day							
0-1	0.00	0.21	0.21	0.49	0.19		
1-2	6.25	7.81	10.25	5.62	10.78		
2-3	18.30	13.40	3.46	38.89	8.79		
3-4	20.20	26.20	14.00	54.74	52.75	71.60	71.75
4-5	4.00	19.70	9.81	6.74	16.60	6.43	
5-6	2.41	7.79	6.42				
6-7	5.15	9.94	5.44				
9-10	4.00	0.98	5.31	1.32	5.69	1.99	
13-14	2.34	1.06	2.23	1.38	2.41		

Figure 5d	(each data point comes from an individual animal)				
MP/AP ratio					
Day post NTX	**0**	**1**	**2**	**3**	**4**
	0.1160903	0.3177383	0.8916606	0.7694048	0.1493902
	0.2427484	0.227777	0.6514887	0.5280196	0.1891386
	0.5204692	0.3856974	0.8560127	0.803865	
	0.3192114		0.7579272	0.366540	
	0.2133863				

Figure 7a	(each data points comes from the average of 3 measurements taken from one experiment)	
% MyHC+ GFP+ cells	**MP-AP**	**MP-MP**
	60.	31.
	60.	34.
	57.	35.
	55.	31.
	77.	34.
	47.	26.

Figure 7b	(each data point comes from the average of 3 replicate PCR reactions, which were derived using cDNA from cells sorted from a minimum of 5 animals)					
expression relative tbp						
AP:MP ratio	**1:1**		**0.5:1**		**0.1:1**	
Myogenin	512.000	560.278	396.177	398.932	250.732	254.232
Mrf4	0.818	1.064	0.521	0.403	0.285	0.321
MyHC	34.776	38.055	24.084	25.813	20.252	15.780

Figure 7c	(each data point from the average of 3 replicate PCR reactions, which were derived using cDNA from cells sorted from a minimum of 5 animals)									
	eMyHC					**Mrf4**				
MPs with AP	8.491	7.313	7.642	7.791	6.540	4.746	6.405	6.551	4.821	7.325
MPs with MP	10.000	9.153	5.686			10.000	8.131	4.593		
	MyoD					**Myogenin**				
MPs with AP	9.692	7.392	9.214	7.131	8.639	10.000	7.068	9.048	7.225	7.341
MPs with MP	4.543	4.383	3.605			6.728	6.535	4.395		
	Pax3					**Pax7**				
MPs with AP	2.978	2.879	3.074	2.321	2.848	6.844	4.092	4.383	4.240	3.911
MPs with MP	6.508	7.077	6.031			8.800	6.949	5.414		

Figure 7d	(each data point comes from a single well)											
Co-culture												
Day	**MP only**				**AP only**				**MP-AP co-culture**			
2.	0.025900	0.018300	0.025800	0.026800	0.002500	0.005900	0.014600	0.010200	0.006400	-0.002400	0.007300	0.004100
3.	0.018125	0.019925	0.021425	0.039625	0.015225	0.019925	0.029625	0.010125	0.015125	0.016125	0.009825	0.014425
4.	0.096750	0.142250	0.146850	0.104950	0.080750	0.099550	0.066050	0.094650	0.068250	0.174750	0.148850	0.120950
5.	0.382400	0.294800	0.308400	0.319600	0.123600	0.117500	0.092400	0.114200	0.306100	0.308200	0.307700	0.265800
7.	1.513625	1.590625	1.647525	1.701525	1.030225	0.899725	1.088425	1.031725	1.864725	1.923425	1.567125	1.931725

Figure 7e	(each data points comes from the average of 3 measurements taken from one experiment)	
%BrdU+ MPs after co-culture	**MP WITH AP**	**MP WITH MP**
	70.909	73.469
	62.500	68.889
	68.085	56.000
		24.138

Supplementary Table 3: Applied Biosystems PCR primer pairs used.

	Taqman Gene Expression Assay
C/EBP alpha	Mm00514283_s1
C/EBP beta	Mm00843434_s1
C/EBP delta	Mm00786711_s1
eMyHC	Mm01332475_g1
FABP4	Mm00445880_m1
GAPDH	Hs02758991_g1
KLF5	Mm00456521_m1
KROX20	Mm00456650_m1
Leptin	Mm00434759_m1
Mrf4	Mm00435126_m1
Myf5	Mm00435125_m1
MyHC	Mm01332493_g1
MyoD	Mm00440387_m1
Myogenin	Mm00446194_m1
Pax3	Mm00435491_m1
Pax7	Mm00834079_m1
PPARg2	Mm00440940_m1
UCP1	Mm00494069_m1