Stem Cell Reports

Supplemental Information

Human Satellite Cell Transplantation and Regeneration from Diverse Skeletal Muscles

Xiaoti Xu, Karlijn J. Wilschut, Gayle Kouklis, Hua Tian, Robert Hesse, Catharine Garland, Hani Sbitany, Scott Hansen, Rahul Seth, P. Daniel Knott, William Y. Hoffman, and Jason H. Pomerantz

Supplemental Figures

Figure S1

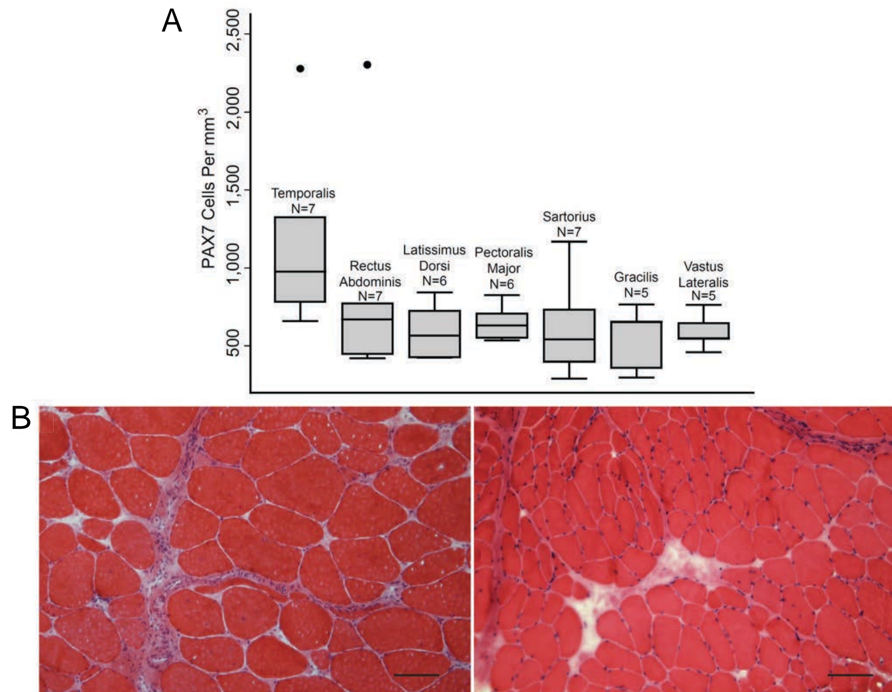


Figure S1 (related to Figure 1): Estimated PAX7 cell content per mm^3 of diverse human skeletal muscles.

A) Box plot represents the number of PAX7 cells per mm^3 volume of tissue for each sample. Boxes are grouped by muscle type. The PAX7 cell content of each sample was calculated by the following formula: PAX7 cells per mm^3 = number of myofibers per mm^2 x PAX7 cell frequency per mm myofiber. Values used in calculation and resulting PAX7 cell content for each sample are shown in Table S1. N=5-7 muscles per group. The middle line of each boxplot indicates the median, the lower and upper lines represent the 25th and 75th percentile, respectively. The outer lower and higher horizontal bars represent the 25th percentile – 1.5 interquartile range and 75th percentile +1.5 interquartile range, respectively. Black dots indicate outlier samples.

B) Representative H&E sections showing variation in number of myofibers per mm^2. Left panel is a representative section of the rectus abdominis muscle of a 56-year-old man. Right panel is

a representative section of the temporalis muscle of a 54-year-old man. Scale bar represents 100μm.

Figure S2

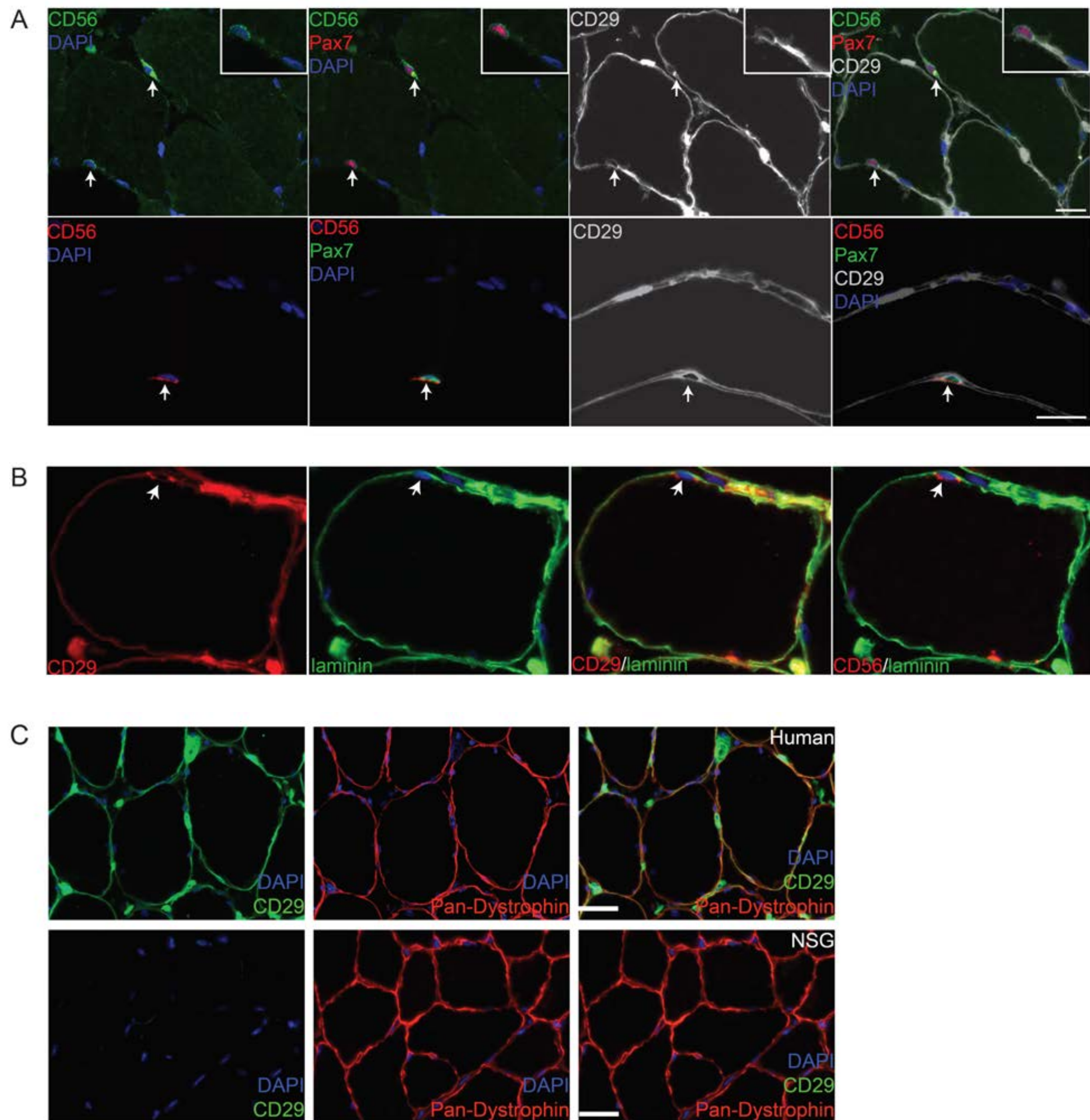


Figure S2 (related to Figure 1): Human PAX7/CD56 positive satellite cells and human skeletal muscle fibers express CD29.

A) Representative sections of human skeletal muscle showing identification of satellite cells using antibodies for PAX7, CD56 and CD29. Top and bottom rows show representative cross and longitudinal sections, respectively. Top row: Two CD56+/PAX7+ satellite cells (arrows top

left panel) are present in the section (first and second panels). Inset shows enlarged image of the satellite cell indicated by lower arrow. Human specific mouse anti-CD29 marks satellite cells, fibers, and interstitial cells. Bottom row: One satellite cell shown on longitudinal section (arrow). CD29 marks adjacent fiber membranes in addition to the satellite cell membrane (right two panels).

B) Representative section demonstrating the relationship between Laminin and CD29 on human satellite cells and fibers. (Left) Human CD29 marks the fiber as well as the circumference of the satellite cell (arrow). (Second panel from left) Laminin marks the basal lamina and clearly shows the sublaminar position of the satellite cell. (Third panel from left) The satellite cell is circumferentially marked by CD29 as opposed to Laminin which labels the basement membrane. (Fourth panel from left) CD56 marks the satellite cell with surface staining that is preferential to the apical surface adjacent to the fiber.

Scale bars – 20μm.

C) Control immunostaining panels confirming human specificity of BioLegend anti-human CD29 antibody TS2/16. Top panels show a section from a human latissimus muscle stained using the BioLegend anti-human CD29 antibody TS2/16 and counterstained with DAPI and pan-Dystrophin. Scale bar – 50μm. Bottom panels show a section from an NSG TA muscle stained with the same antibodies. Scale bar - 25μm. The staining for each sample was done side-by-side and the images were taken at the same time with identical exposures and image processing.

Figure S3

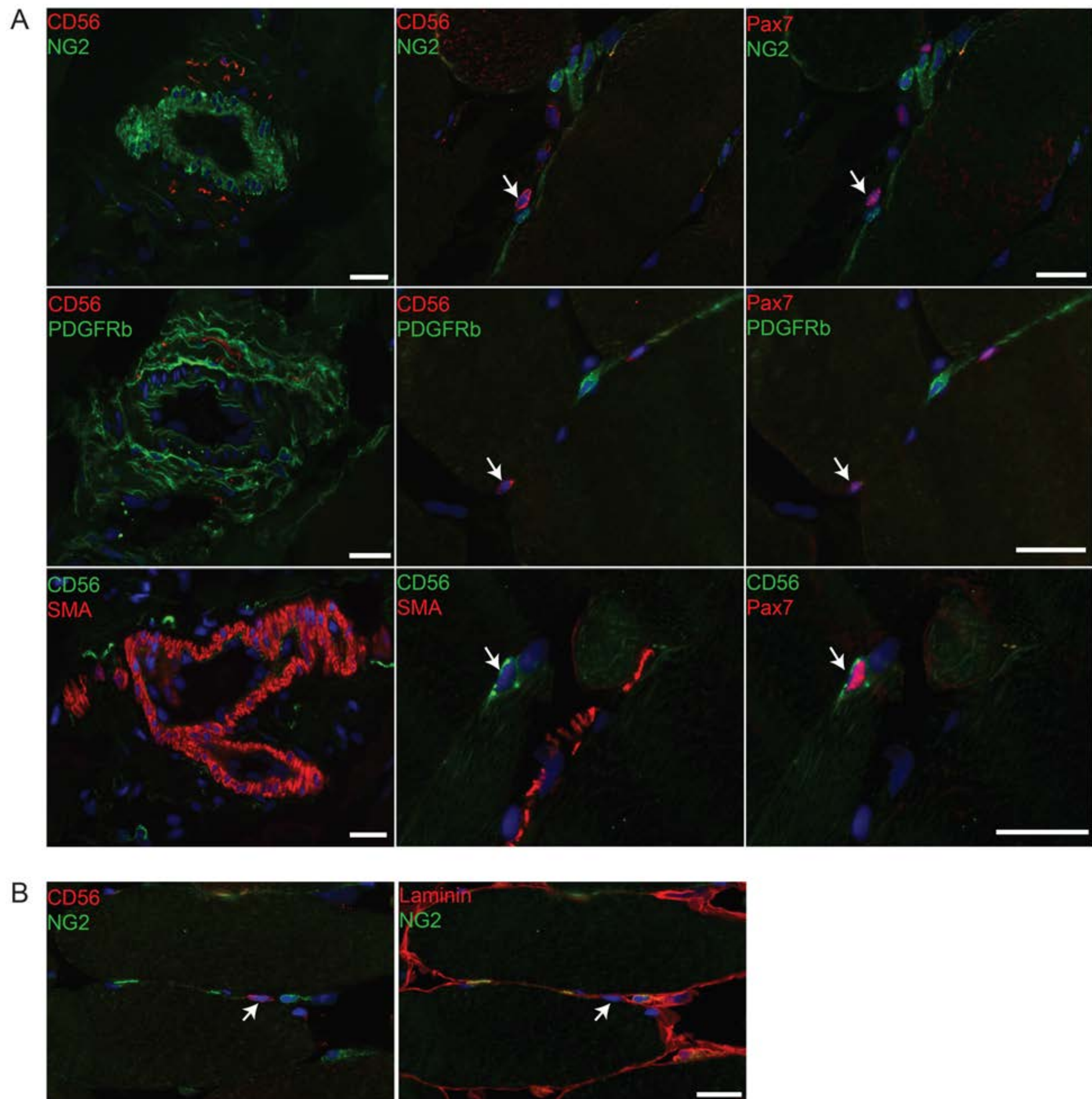


Figure S3 (related to figure 1): Satellite cells are distinct and distinguishable from pericytes and vessel associated cells.

A) Representative sections of human skeletal muscle showing the relationship of CD56+/PAX7+ satellite cells to cells expressing various pericyte markers. Top row: NG2**.** Left – typical NG2 staining pattern in a vessel within human muscle. Middle and right – CD56+/PAX7+ cell (arrow)

does not express NG2 in contrast to the NG2+ cells on the section that do not express CD56 or PAX7. Middle row: PDGFRb. Left - typical PDFRb staining pattern in a vessel within human muscle. Middle and right – CD56+/PAX7+ cell (arrow) does not express PDGFRb in contrast to the PDGFRb+ cells on the section that do not express CD56 or PAX7. Bottom Row: SMA. Left - typical SMA staining pattern in a vessel within human muscle. Middle and right – CD56+/PAX7+ cell (arrow) does not express SMA in contrast to the SMA+ cells on the section that do not express CD56 or PAX7.

B) Representative section confirming sublaminar location of CD56+ satellite cell (arrow) that does not express NG2, in contrast to the extralaminar NG2 cells which are CD56 negative.

Scale bars – 20μm.

Figure S4

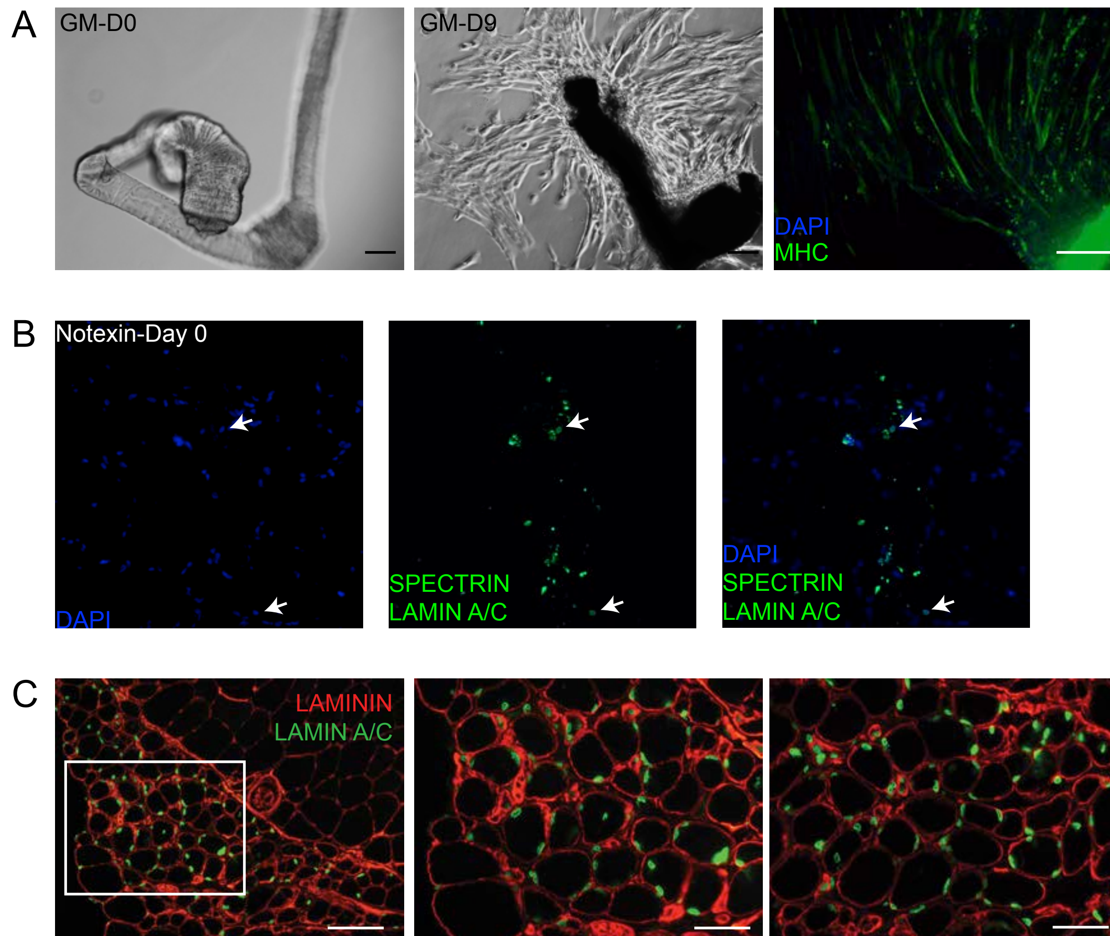


Figure S4 (related to Figure 2): Characterization of progeny cells from isolated live human muscle fibers in culture and after transplantation

(A) Left - Phase image of a single live myofiber from the rectus abdominis of a 64-year-old woman immediately after isolation. Middle - Phase image of a contracted single myofiber in GM for 9 days with multiple progeny cells. Right - Immunostaining for myosin heavy chain (MHC) in progeny cells from a single myofiber after 7 days in DM. GM - growth medium; DM - differentiation medium. Scale bars – 20μm

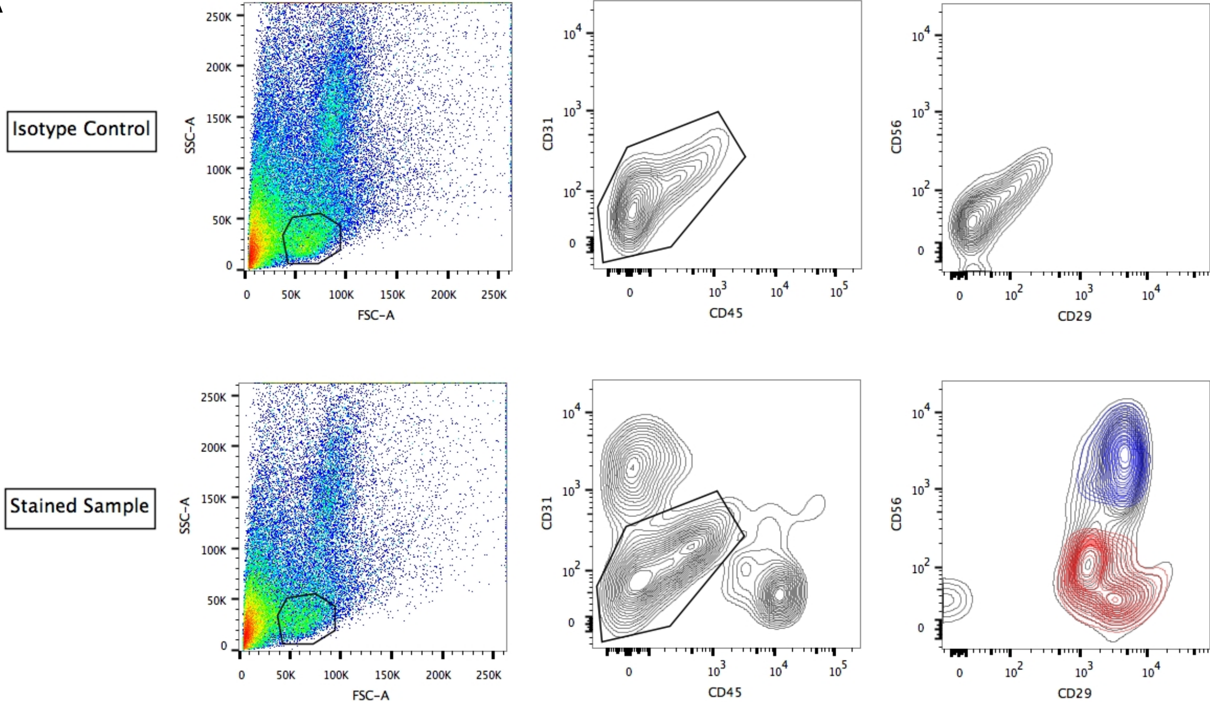
(B) Separate channels shown for Figure 2B left hand panel to facilitate identification of human nuclei at Day 0. Two examples of human LAMIN A/C positive, DAPI positive nuclei are marked

with arrows. Others can be readily identified in this section. The remainder of the green staining that is non-nuclear is specific SPECTRIN / LAMIN A/C staining of cell fragments.

(C) Images of adjacent sections to those shown in Figure 2C, right, stained for LAMININ and human LAMIN A/C. Left – Area of human cell engraftment. White box indicates field shown in middle panel. Scale bar – 200µm. Middle – Higher magnification image showing human derived nuclei within and outside of the LAMININ marked basal lamina. Scale bar – 100µm. Right – A second representative area. Scale bar – 100µm. Of a total of 227 nuclei counted from four different fields, 82% of the human nuclei are positioned within the basal lamina, and 18% are located in the interstitial space.

Figure S5

A



B

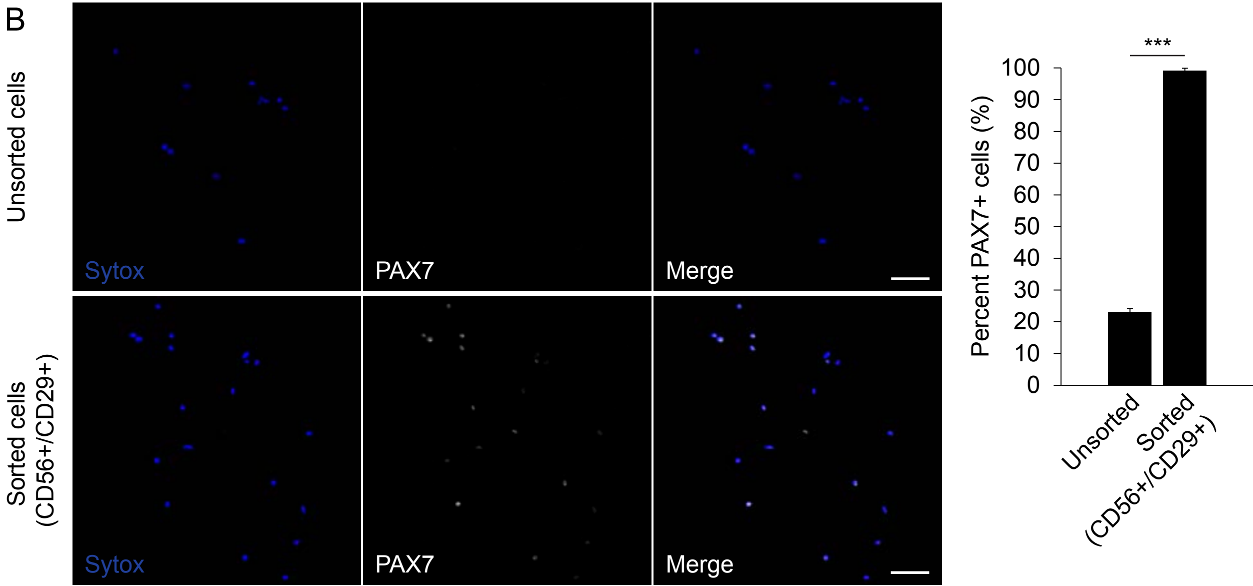


Figure S5 (related to Figure 4) Isotype controls for FACS sorting, and Pax7 immunostaining of sorted CD56/CD29 cells.

A) Top panels show isotype controls. Non-specific binding is insignificant. Lower panels – same plots as in Figure 4 for reference.

B) Pax7 immunostaining of sorted CD56/CD29 cells and unsorted cells. Cells were prepared from the vastus lateralis muscle of an 84 year old male. Top row - cells collected after muscle sample digestion prior to sorting (unsorted cells) Bottom row – CD56+/CD29+ sorted cells. All cells were counterstained with Sytox. Anti-Pax7 antibody was purified from hybridoma (DSHB) supernatant and directly conjugated using Alexa-fluor-350. Confocal images were taken and pseudocolored. Scale bar 50μm. Right – Percentage of cells in each sample that stained positive for Pax7. Student's t-test was used for statistical analysis of 20 fields counted for each condition.

Figure S6

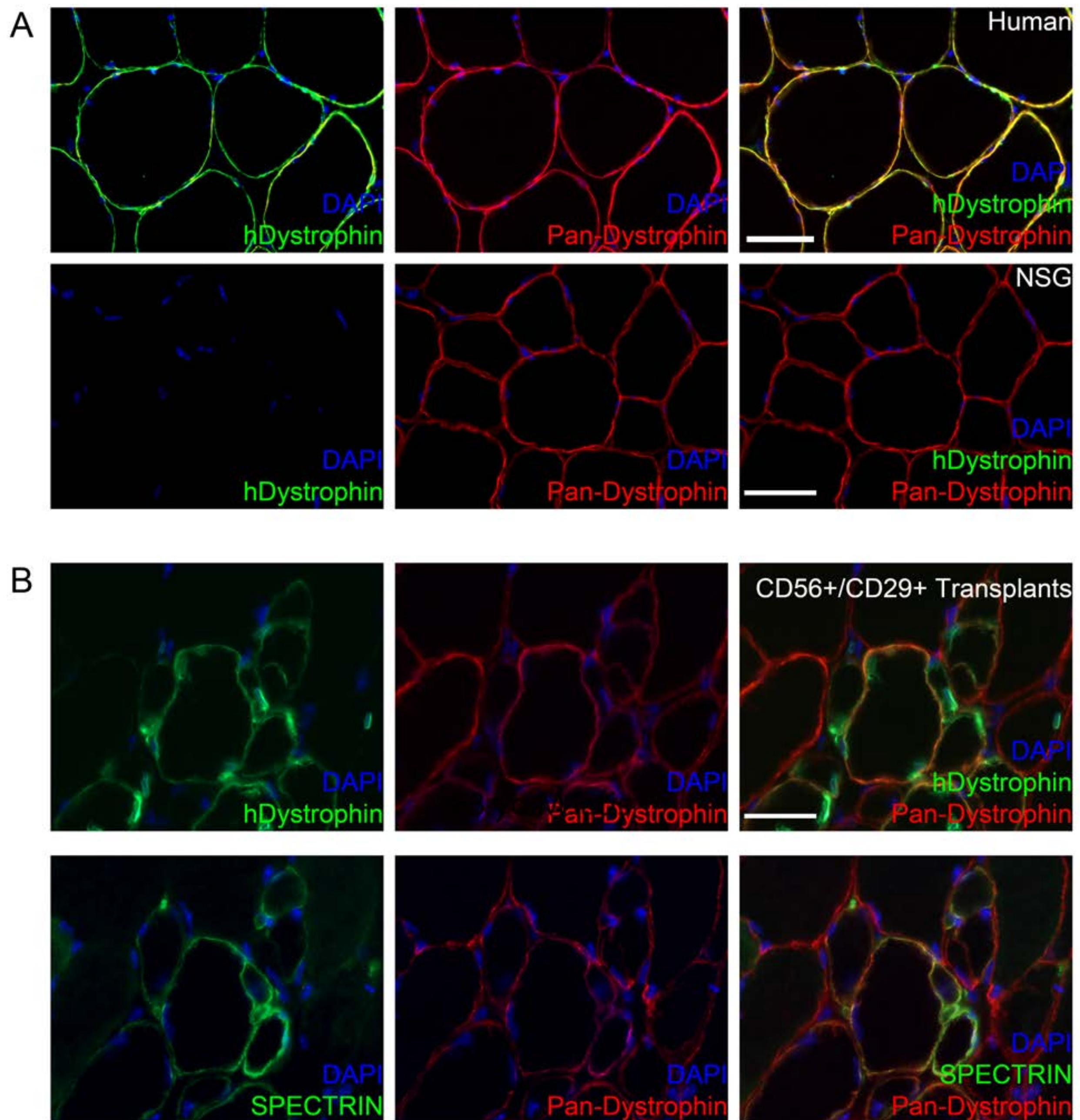


Figure S6 (related to Figure 5): Antibody controls.

A) Controls for human specificity of Leica DYS3 anti human Dystrophin**.** Immunostained frozen sections of Top - latissimus dorsi muscle (83 y/o male) and Bottom – NSG mouse TA muscle.

Pan – Dystrophin used as counter stain to show presence of muscle fibers. Scale bar top 50μm, bottom 25μm.

B) Comparison of human Dystrophin staining pattern with Spectrin on adjacent sections. Adjacent sections were taken from a TA muscle harvested 5 weeks after transplantation with 5,000 CD56+/CD29+ cells (same experiment as shown in Figure 5B). Top row- section stained with human and pan dystrophin antibodies. Bottom row – adjacent section stained with human Spectrin antibody and pan-dystrophin.

Figure S7

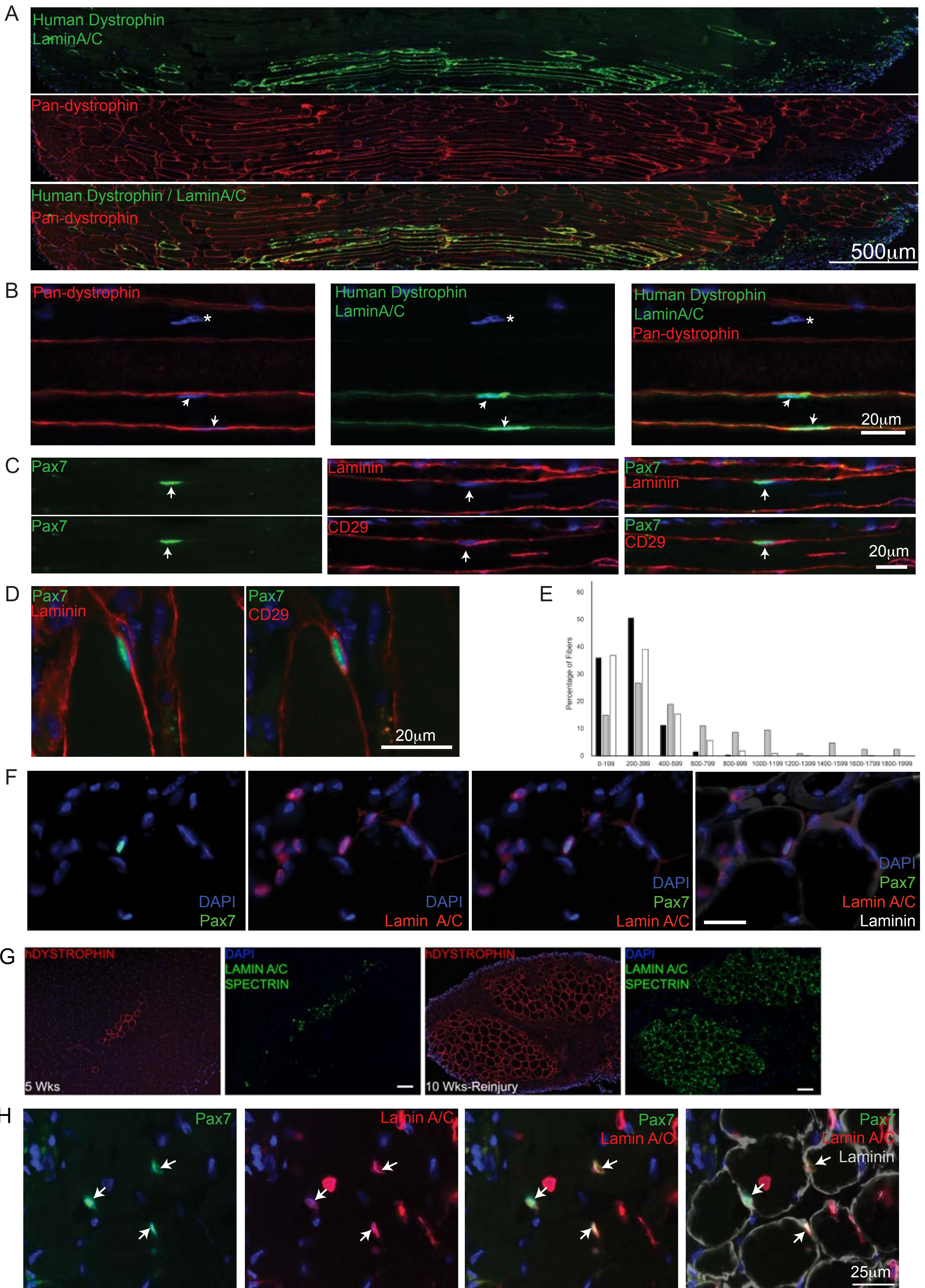


Figure S7 (related to Figure 5): Analysis of human derived fibers.

A) Representative longitudinal section of a re-injured muscle evaluated 5 weeks after re-injury and 10 weeks after human muscle stem cell transplantation. Top panel: staining for human Dystrophin and Lamin A/C. Human-derived muscle fibers were evaluated in continuity longitudinally in TA muscle. Long sections of human-derived fibers are apparent in which the great majority of nuclei are human-derived. The upper portion of the image shows an area without human cell engraftment. Middle panel: staining for pan-dystrophin detects fibers of both mouse and human origin. Bottom panel: merged image. The images are a composite of adjacent stitched images from the section.

B) Higher power representative images distinguishing mouse from human cells and nuclei. Mouse derived nucleus (asterisk) is negative for Lamin A/C and is present in a region that is negative for human Dystrophin. Human derived nuclei (arrows) express Lamin A/C and are within a fiber expressing human Dystrophin. Blue - DAPI

C) PAX7 satellite cells are human derived. Left – a PAX7 positive cell is shown. Middle – The sublaminar position is confirmed by Laminin staining (top) and human origin of the fiber region and the satellite cell (arrow) by human CD29 staining (bottom). Right – Merged images. The distinction between laminin staining of the basement membrane indicating the sublaminar position of the satellite cell and circumferential CD29 staining of the satellite cell is evident.

D) Additional example of a human derived satellite cell after reinjury. The distinct patterns of Laminin and human CD29 staining together confirm the human origin and the sublaminar position of the cell.

E) Comparison of fiber areas of engrafted human myofibers after human CD56+/CD29+ cell transplantation. The area of all human SPECTRIN-positive muscle fibers in irriadiated NSG TA after transplantation with 5,000 CD56+/CD29+ cells from the latissimus dorsi muscle of a 64-year-old man was measured at 5 weeks (black bar), 10 weeks (gray bar) and 10 weeks, notexin

reinjury (white bar). The y axis is the percent of human fibers, the x-axis is the range of fiber area (um^2). Each bar represents the percent of engrafted human fibers of the transplant group that falls in the area range. Measured average NSG host- muscle fiber area is 2273.6±833.6um^2, measured average muscle fiber area of the same donor muscle is 5752.3±1330.5um^2. Average fiber area for 5 weeks is 253.9±128.8um^2, average fiber area for 10 weeks is 611.0±454.5um^2, average fiber area for 10 weeks-reinjury is 303.3±205.4um^2.

F) Example of human satellite cell identified by co-staining with Pax7 and human Lamin a/c. Section is from TA muscle harvested at 5 weeks after transplantation of 5,000 CD56+/CD29+ human cells (from experiment shown in figure 5B). Laminin staining was used to confirm sublaminar position of the Pax7 human cell. Scale bar 25μm.

G) Correlation of human Dystrophin expression with human Spectrin at 5 weeks and after re-injury (10 weeks). Adjacent sections to those shown in Figure 5B and C were stained with human Spectrin antibody. Scale bar 100μm.

H) Satellite cells within the transplanted muscle after reinjury (10 weeks). Sections were immunostained for PAX7, human Lamin A/C and Laminin. Representative area shows sublaminar PAX7 cells that express human Lamin A/C (arrows). A centrally located Lamin A/C nucleus that does not express PAX7 is visible as well. 70/70 analyzed PAX7 cells expressed Lamin A/C and host PAX7 satellite cells were not detected either within or outside of areas of human cell engraftment.

Supplemental Tables

Table S1: Analysis of PAX7 cells of diverse human muscles (related to Figure 1).

Muscle	Donor demographic	Number of fibers analyzed	Length of fibers analyzed (mm)	Number of PAX7 cells counted	Number of fibers per mm^2	Number of PAX7 cells per mm^3
Rectus Abdominis	40 F	10	57.6	225	197.3	771
Rectus Abdominis	45F	11	75.6	197	284.0	740
Rectus Abdominis	52 F	10	76.5	228	218.7	652
Rectus Abdominis	74M	10	199.8	333	401.6	669
Rectus Abdominis	56M	10	56.7	206	122.7	446
Rectus Abdominis	28F	11	174.9	620	649.6	2303
Rectus Abdominis	44F	10	67.2	806	35.1	421
Sartorius	62M	14	1138.6	194	2350.8	401
Sartorius	69M	10	1308.6	130	2931.2	291
Sartorius	82F	10	75.6	213	260.0	732
Sartorius	72M	10	80.4	266	164.0	543
Sartorius	85M	10	80.85	208	154.7	398
Sartorius	70F	11	80.55	258	218.7	700
Sartorius	48F	10	117.9	393	350.2	1167
Vastus Lateralis	59M	10	77.1	229	217.3	645
Vastus Lateralis	56F	10	63.75	158	220.0	545
Vastus Lateralis	66M	10	61.2	124	226.7	459
Vastus Lateralis	55F	10	64.2	167	210.7	548
Vastus Lateralis	46F	10	72.6	215	257.3	762
Pectoralis Major	55F	10	54.6	103	293.3	553
Pectoralis Major	49F	10	60	98	371.9	607
Pectoralis Major	43F	10	61.8	131	389.4	825
Pectoralis Major	44F	10	107.4	238	318.7	706
Pectoralis Major	44F	10	97.8	163	320.7	534
Pectoralis Major	51F	10	143.4	348	269.3	654
Latissimus Dorsi	68M	30	172.2	316	232.0	426
Latissimus Dorsi	50F	10	71.7	267	172.0	641
Latissimus Dorsi	83M	10	86.1	211	173.3	425
Latissimus Dorsi	39F	10	61.2	209	144.0	492
Latissimus Dorsi	60M	10	106.8	397	226.7	843
Latissimus Dorsi	54M	10	111	351	229.2	725
Gracilis	11F	10	140.7	237	213.3	359
Gracilis	65M	10	176.1	277	189.3	298
Gracilis	72M	10	88.8	177	328.0	654
Gracilis	45F	10	89.4	409	142.7	653
Gracilis	72M	10	129.6	509	194.7	765
Temporalis	19F	10	47.4	155	238.7	780
Temporalis	33M	10	75	254	288.0	975
Temporalis	27F	10	69	274	333.3	1324
Temporalis	33M	10	105	324	357.4	1103
Temporalis	58M	10	78.6	714	250.7	2277
Temporalis	54M	10	77.4	283	180.0	658
Temporalis	44M	10	57.6	206	228.2	816

The human muscle type and the donor's age and sex for each sample analyzed for PAX7 frequency are shown along with the number of fibers analyzed, their aggregate length and the number of PAX7 cells identified. Fiber content (number of fibers per mm) and the PAX7 cell content (number of PAX7 cells per mm^3) for each sample are also shown. Satellite cell content is calculated by the formula: PAX7 cells per mm^3 = number of myofibers per mm^2 x PAX7 cell frequency per mm myofiber.

Table S2: Frequency of PAX7, CD56 and CD29 cells along a fiber of different human muscles (related to Figure 1).

	Number of PAX7 cells counted/mm of fiber analyzed	Number of CD56 cells counted/mm of fiber analyzed	Number of CD29 cells counted/mm of fiber analyzed	PAX7 cells per mm	CD56 cells per mm	CD29 cells per mm
Rectus Abdominis, 52F	288/76.5	207/73.8	298/86.4	2.98	2.8	3.45
Rectus Abdominis, 28F	620/174.9	293/85.5	290/70.8	3.54	3.43	4.1
Latissimus Dorsi, 83M	211/86.1	207/102.3	312/90.6	2.45	2.49	3.44
Latissimus Dorsi, 54M	351/111	246/72.6	252/66	3.18	3.05	3.92
Sartorius, 48F	393/117.9	348/114	275/70.2	3.33	3.39	3.82
Vastus Lateralis, 56F	158/63.7	217/84.6	239/82.2	2.48	2.57	2.91

A subset of muscle samples were stained separately for PAX7, CD56 and CD29. The muscle type, and the donor's age and sex for analyzed samples are shown. For each sample, a total of 30 fibers was analyzed (10 fibers analyzed for PAX7, 10 fibers analyzed for CD56 and 10 fibers analyzed for CD29). The aggregate length of the fibers analyzed as well as the number cells positive for each marker is shown. Right 3 columns show the frequency of PAX7, CD56 and CD29 cells for each sample.

Table S3: Comparison of estimated and predicted human satellite cell content on isolated myofibers (related to Figure 2).

Aggregate length of fibers transplanted (mm)	15
Theoretical PAX7 frequency (cells/mm)	4.7
PAX7 Cell Count Fiber 1	18
PAX7 Cell Count Fiber 2	12
PAX7 Cell Count Fiber 3	9
PAX7 Cell Count Fiber 4	15
PAX7 Cell Count Fiber 5	17
PAX7 Cell Count Fiber 6	14
PAX7 Cell Count Fiber 7	11
PAX7 Cell Count Fiber 8	12
PAX7 Cell Count Fiber 9	15
PAX7 Cell Count Fiber 10	9
PAX7 Cell Count Fiber 11	9
Actual number of PAX7 cells transplanted	70
Theoretical number of PAX7 cells transplanted	64

A rectus abdominis biopsy from a 64-year-old woman was digested with collagenase and single myofibers were isolated. Five fibers of equal length were transplanted (results shown in Table 1). Eleven fibers of equal length as transplanted fibers were fixed and stained for PAX7 and LAMININ and the number of sublaminar PAX7-positive nuclei were counted (representative image shown in Figure 2B). The PAX7 count for each fiber is listed and the estimated number of satellite cells transplanted is determined by the average of the satellite cells counted from those 11 fibers multiplied by 5. The predicted satellite cell content is determined by the average PAX7 cell frequency per mm fiber (Table S1) multiplied by aggregate fiber length transplanted (15mm).

Table S4: Comparison of theoretical human satellite cell yield and actual human satellite cell yield after FACS sorting (related to Figure 4).

Muscle	Sort time after biopsy	Sample Volume (cm^3)	Actual CD56+/CD29+ cell yield	Theoretical CD56+/CD29+ cell yield
Rectus Abdominis, 64M	Day 1 After Biopsy	1.02	7,389	800,000
Vastus Lateralis, 81M	Day 1 After Biopsy	5	73,833	2,960,000
Vastus Lateralis, 69M	Day 1 After Biopsy	6	103,586	3,552,000
Vastus Lateralis, 40M	Day 1 After Biopsy	8	118,969	4,736,000

The volume of muscle tissue from 4 different muscles from 4 individuals was measured before the samples underwent digestion for FACS sorting. The total number of live CD56+/CD29+ cells obtained during the sort was recorded (actual cell yield) for each sample. To calculate the theoretical yield, the volume of each muscle was multiplied by the average of the measured satellite cell content (Table S1) for that muscle (theoretical cell yield).

Supplemental Experimental Procedures

Animal Care and Transplantation Studies

All mice were bred and housed in a pathogen-free facility at the University of California San Francisco. All procedures were approved and performed in accordance with the University of California San Francisco Institutional Animal Care and Use Committee. All experiments were performed in NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (The Jackson Laboratory). NSG mice were pretreated with 18 gamma (Gy) or 12 Gy radiation on the day of transplantation. NSG mice exposed to 18Gy limited to the hindlimb occasionally developed ulcerations of the skin. These were treated with ointment per UCSF LARC protocols. A 1 cm incision was made in the mouse skin overlying the TA muscle and human cells or myofiber preparations were injected along with notexin (0.1ug in 50uL HBSS) (Latoxan) or 50ul 0.5% bupivacine directly into the muscle of one leg. For cell injection a 27 ½ gauge needle on a 1 cc syringe was used and for fiber transplantation a 21 gauge needle on a 1 cc syringe was used. The skin was closed with sutures and skin glue was applied over the incision. Transplanted TA muscles were harvested immediately, 1 week, 5 weeks or 10 weeks after transplantation. At 5 weeks post-transplant, some mice were reinjured with notexin (0.1ug) (Latoxan) and analyzed 5 weeks post reinjury. Harvested muscles were frozen in isopentane chilled in liquid nitrogen. Serial 10 μm transverse sections of the whole muscle were analyzed.

Human Myoblast Preparation

Frozen human myoblasts (Gibco, Cat#A12555) were thawed and plated onto 0.1% gelatin coated tissue culture treated flasks in growth medium (DMEM, 20% FBS, l-glut, sodium pyruvate, supplemented with antibiotics) containing 5ng/ml basic fibroblast growth factor (R&D Systems, Minneapolis, MN). Medium was changed every two days until the myoblasts reached an 80% confluence after which the myoblasts were passaged. Myoblasts were transplanted at p2, mpd 13 - 14.

Human Muscle Analysis

After procurement, human muscle samples were split for analysis. Part of the specimen was immediately fixed in 4% paraformaldehyde (PFA) at room temperature for 10 min and washed with PBS. The sample was then embedded in glycerol. For this, the sample was first placed in 30% glycerol in PBS overnight at 4°C, then 50% glycerol in PBS overnight at 4°C, then 80% glycerol in PBS overnight at 4°C and finally 100% glycerol. The fibers are stored in 100% glycerol at 4°C until dissection.

Single fibers were dissected using fine forceps under a dissecting microscope. Single fibers were washed in PBS for 15 min at room temperature, then permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 6 min, and washed with PBS for 15 min. Single fibers were then blocked with 10% goat serum in PBS for 30 min at room temperature and incubated overnight at 4°C with the following primary antibodies: mouse monoclonal anti-PAX7 (1:25 Developmental Studies Hybridoma Bank, Cat# PAX7), anti-human CD29 (1:25, Serotec Cat# MCA2028) (Figure 1), or FITC-conjugated anti-CD56 (1:25 NCAM16.2, BD Biosciences, Cat# 340410) and rabbit polyclonal anti-LAMININ (1:25, Sigma-Aldrich Cat# L9393) diluted in PBS containing 10% normal goat serum. After PBS wash at room temperature for 15 min, the following corresponding secondary antibodies were applied for 1 hr at room temperature: Alexa Fluor 488 goat anti-mouse IgG (1:500 Invitrogen, Cat# A-11001), Alexa Fluor 555 goat anti-rabbit IgG (1:500Invitrogen, Cat# A-21428). Fibers were washed in PBS for 15 min twice and then mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories Cat# H-1200) and all samples were examined using a Leica upright microscope.

The remaining portion of the sample that was not fixed was frozen in isopentane chilled in liquid nitrogen. Transverse 6 or 10 μm cross sections were obtained. Sections were thawed at room temperature for 15 min.

For hematoxylin and eosin (H&E) staining, slides with 10 μm cross sections were rinsed with water and dried. Sections were then dehydrated as follows; 5 min in xylenes, twice; 5 min in 100% ethanol, twice; 5 min in 95% ethanol; 5 min in 80% ethanol and washed in water for 30 s. Slides were then placed in 3x Gill's Hematoxylin for 4 min and washed with water for 30 s. For nuclear staining, slides were placed in Scott's water for 3 min and washed in water for 30 s. Slides were then placed in Eosin for 2 min and washed with water for 30 s. Sections were dehydrated again as follows: 1 min 80% ethanol; 2 min 95% ethanol, twice; 3 min 100% ethanol, twice; 2 min xylenes, twice. Slides were then mounted with Permount and coverslips and air dried overnight before imaging. All samples were examined using a Leica upright microscope and muscle area was analyzed using ImageJ (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014).

For antibody staining the slides with 6-10 μm cross sections were fixed in 2-4% PFA at room temperature for 5-10 min, washed in PBST (PBS+ 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO)), blocked with DAKO Protein Block (Cat# X0909) and incubated at room temperature overnight with the following primary antibodies: mouse monoclonal anti-PAX7 (1:25 Developmental Studies Hybridoma Bank, Cat# PAX7), Alexa Fluor 647-conjugated anti-human CD29 TS2/16 (1:50 BioLegend, Cat# 303018) (figures 3, 4G, S2, S7), rabbit monoclonal anti-CD56 (1:100 abcam, Cat# ab75813) (Figures 1, S2 top and 3C), mouse monoclonal anti-CD56 (1:10 BD Biosciences Cat# 555515) (Figure S3), rabbit polyclonal anti-MYOD (1:200, Santa Cruz Biotechnology, Cat# sc-760), rabbit polyclonal anti-MYF5 (1:200 Santa Cruz Biotechnology, Cat# sc-302), mouse monoclonal anti SMA (1:400 Sigma Cat# C6198), rabbit anti PDGFRb (1:100 Cell Signaling Cat# 3169), rabbit anti NG2 (1:100 Millipore Cat# AB5320),

rabbit polyclonal anti-LAMININ (1:25 Sigma-Aldrich, Cat# L9393) or chicken polyclonal anti-LAMININ (1:1000 abcam, Cat# ab14055,) diluted in DAKO Antibody Diluent (DAKO Cat# S0809). After PBST wash for 3 min 3 times, the following corresponding secondary antibodies were applied for 1 hr at room temperature: FITC-conjugated donkey anti-mouse (1:300 Jackson Immunology,Cat# 715-095-150), Cy3-conjugated donkey anti-mouse (1:500 Jackson Immunology, Cat# 715-165-150), Cy5-conjugated donkey anti-chicken (1:200 Jackson Immunology, Cat# 703-175-155), Cy5 donkey anti-mouse (1:200 Jackson Immunology, Cat# 715-175-150), FITC-conjugated donkey anti-rabbit (1:300 Jackson Immunology, Cat# 711-095-152) diluted in DAKO Antibody Diluent (Carpinteria Cat# S0809). Sections were mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories Cat# H-1200) and all samples were examined using a Leica upright microscope.

Isolation of Live Human Muscle Fibers

Immediately after human muscle was explanted, the sample was incubated for 1 hr in 0.2% type I collagenase (Sigma-Aldrich, St. Louis, MO) in DMEM with sodium pyruvate and placed in a shaker in a 37°C incubator. The sample was then transferred to a petri dish coated with horse serum and then washed in DMEM with sodium pyruvate supplemented with antibiotics. After wash, the sample was placed in a fresh horse-serum-coated petri dish with DMEM with sodium pyruvate supplemented with antibiotics and individual fibers were separated under a dissecting microscope using fine forceps and fiber length was measured. 5 dissected single fibers were then taken up in a 1 cc syringe with a 21 gauge needle and injected into the recipient muscle with the appropriate myotoxin. Before fibers were aspirated, 10% horse serum was passed through the needle and syringe to prevent fiber adhesion. Single fibers that were not transplanted were either fixed with 4% PFA or cultured. For fixation, dissected fibers were taken up in a 1 cc syringe with a 21 gauge needle and injected into 4%PFA and the fixed fibers were stained for PAX7 and LAMININ as described above for glycerol-embedded fibers.

For live fiber culture, individual fibers were aspirated and injected into culture media (20%FBS, 10% HS in DMEM with sodium pyruvate supplemented with antibiotics). Culture medium was changed every 2 days. After being in culture medium for 2 weeks, wells were either fixed with 4% PFA or switched to differentiation medium (DMEM F12, 2% HS) for 7 days. Differentiation medium was changed daily. After 7 days, cells were fixed with 4% PFA. All fixed cells were washed with PBS for 15 min and then permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 6 min. After PBS wash for 15 min they were blocked with 10% goat serum in PBS and incubated for 1 hr at room temperature with the following primary antibodies diluted in 10% goat serum in PBS: mouse monoclonal anti-myosin heavy chain (1:100 Novus Biologicals, Cat# NB300-284, Littleton, CO) or rabbit polyclonal anti-MYOD (1:200 Santa Cruz Biotechnology, Cat# sc-760). Cells were then washed in PBS for 15 min and the following secondary antibodies diluted in 10% goat serum in PBS were applied for 1 hr at room temperature: Alexa Fluor 488 goat anti-mouse IgG (1:500, Invitrogen, Cat# A-11001) or Alexa Fluor 555 goat anti-rabbit IgG (1:500, Invitrogen Cat# A-21428). Wells were mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Cat# H-1200) and all samples were examined using a Leica inverted microscope.

Cell preparation from human muscle biopsies

Freshly harvested human muscle was either immediately digested or stored overnight in 30% FBS at 4°C. At the time of digestion, samples were finely minced with scalpels and incubated in Pronase (1mg/ml, Sigma-Aldrich, St. Louis, MO) in 1% HEPES for 1 hr in a shaker in a 37°C incubator. The tissue was triturated and then centrifuged at 150 g for 2 min. The supernatant was collected and washed in 20% FBS in DMEM. The cells were then centrifuged at 1000 g for 5 min at 4°C. The supernatant was then discarded and the cell pellet stored on ice in 20% FBS in DMEM.

Undigested tissue after Pronase digestion underwent a second digestion in Collagenase XI (1mg/ml Sigma-Aldrich, St. Louis, MO) in 5%FBS in DMEM for 30 min on a shaker in a 37°C incubator. After collagenase digestion, the tissue was triturated and centrifuged at 100g for 2 min. The supernatant was then collected and washed with 20%FBS in DMEM and centrifuged at 1000g for 5 min at 4°C. The supernatant is then discarded and cell pellet is reconstituted with 20% FBS in DMEM and combined with cells from the Pronase digestion

The cells were then filtered through a 100 μM cell strainer followed by filtration through a 40μM cell strainer. The cells were then centrifuged at 1000g for 5 min at 4°C. The supernatant was discarded and the cell pellet underwent erythrocyte lysis by incubation with ACK buffer with 5% FBS for 8 min on ice. After lysis, the remaining cells were washed with cold PBS. If erythrocytes were still seen, the cells underwent a second round of erythrocyte lysis. After wash in PBS the cells were centrifuged at 1000g for 5 min at 4°C. The supernatant was aspirated and the cells were taken up in FACS buffer (HBBS, 2% FBS and 2 mM EDTA (Sigma-Aldrich, St. Louis, MO)).

Some digested human muscle cells were then stained with an antibody cocktail consisting of Pacific Blue-conjugated anti-human CD31 (1:25 BioLegend Cat# 303114), Alexa Flour 700-conjugated anti-human CD45 (1:50 BioLegend, Cat# 304024), Alexa Fluor 488-conjugated anti-human CD29 (1:20 BioLegend, Cat# 303016), and PE-conjugated anti-human CD56 (1:10 MY31 BD Biosciences Cat# BD347747) for 45 min on ice. The remaining cells were stained with the following isotype control antibodies for gating: FITC-conjugated anti-mouse IgG1 (1:20 BioLegend, Cat# 406605), PE-conjugated anti-mouse IgG2 (1:10 MY31 BD Biosciences Cat# BDB550085), Alexa Fluor 700-conjugated anti-mouse IgG1 (1:50 BioLegend, Cat# 400144) and Pacific Blue-conjugated anti-mouse IgG1 (1:25 BioLegend, Cat# 400151San Diego, CA). After antibody incubation, the cells were washed with cold FACS buffer and centrifuged at 700g for 5 min at 4°C for three times. After the last wash the cells were taken up in 200μl FACS buffer with 7AAD (1:1000 BioLegend, San Diego, CA)

Satellite cell sorting

All flow cytometry analysis and cell sorting were performed at the University of California San Francisco Flow Cytometry Core with the BD FACSAria2 operated using FACS Diva software. Viable cells were gated using 7AAD and singlet cells were based on scattering to avoid cell clusters. First, cells incubated with isotype antibodies were analyzed to determine gating. Then viable cells were depleted for CD45 and CD31 expressing cells. Cells that remained after depletion were sorted for CD56+/CD29- and CD56+/CD29+ and collected for further experimentation. Cells to be transplanted were taken up in 50ul of 20%FBS in DMEM supplemented with 10 μM Rho-associated protein kinase inhibitor (ROCKi).

Pax7 immunostaining of cells from digested muscle

Anti-Pax7 antibody was purified by protein A –sepharose affinity from Pax7 hybridoma (DSHB) supernatant using standard protocols. The Pax7 antibody was directly conjugated using Alexa-fluor-350 carboxylic acid succinimide (Life Technologies). Sorted cells were collected in 20%FBS-DMEM with 10 mM ROCKi and plated directly into wells of BioCoated laminin-coated chamber slides (BD Biosciences) previously coated for 1 hr with extracellular matrix gel (1:100, Sigma-Aldrich) in F12/DMEM. The cells were incubated in the chamber slides for 1 hr at 37°C with 5% CO2 to allow for attachment. The cells were washed with PBS and then fixed in 4% PFA for 20 min. The fixed cells were place in pre-heat (95°C) target retrieval solution (Dako) for 20 min then allowed to cool to room temperature. Cells were blocked with 5% Goat Serum in 0.3% Triton X-PBS and incubated with directly conjugated anti-Pax7-Alexa fluor-350 O/N. Cells were counterstained with Sytox-orange (Life Technologies) and mounted in Vectashield without DAPI. Photos were taken on confocal microscope and pseudocolored.

qRT-PCR

RNA was extracted from cells using the RNeasy Mini Kit including DNase treatment (Qiagen, Valencia, CA) according to the manufacturer's instruction. RNA concentration was measured using a Nanodrop ND-1000 Spectrophotometer (Thermo, Waltham, MA) to convert equal quantities of mRNA into cDNA using the Superscript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Relative gene expression was determined using TaqMan Assay (Applied Biosytems, Carlsbad, CA) on an ABI 7300 Real-Time PCR system with the human primer pair PAX7 (Hs00242962m1). Cycle threshold (Ct) value for detecting a gene of interest was normalized against Ct value of the housekeeping gene, human GAPDH (Applied Biosystems, Cat# 4333764F), and relative changes were calculated according the ΔΔCt method.

NSG TA analysis

All glass slides were removed from -80°C and warmed at room temperature for 10 min. H&E staining was performed as described above for human sections.

For human LAMIN A/C and human SPECTRIN immunostaining, NSG sections were fixed in 4% PFA for 10 min at room temperature and then washed with PBS for 3 min 3 times and then permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 6 min. The sections were then washed for 3 min with PBS 3 times and blocked with 10% goat serum in PBS for 30 min at room temperature. The sections were then incubated for 1 hr at room temperature with the following primary antibodies: mouse monoclonal anti-human SPECTRIN (1:100 Leica Microsystems, Cat# NCL-SPEC1 Buffalo Grove, IL) and mouse monoclonal anti-human LAMIN A/C (1:100 Vector Laboratories, Cat# VP550 Burlingame, CA) in 10% normal goat serum in PBS. The sections were then washed for 3 min with PBS 3 times followed by 30 min of incubation at room temperature with Alexa Fluor 488 goat anti-mouse IgG (1:500, A-11001 Invitrogen, Carlsbad, CA) in 10% normal goat serum in PBS. Sections were mounted with

VECTASHIELD mounting medium with DAPI (H-1200 Vector Laboratories, Burlingame, CA) and all samples were examined using a Leica upright microscope.

For all other immunostainings, the slides were fixed in 2% PFA at room temperature for 10 min, washed in PBST (PBS+ 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO)), and then blocked with DAKO Protein Block (X0909 Carpinteria, CA) and incubated at room temperature overnight with the following primary antibodies: mouse monoclonal anti-PAX7 (1:20 DHSB, Iowa City, Iowa), chicken polyclonal anti-pan laminin (1:25 abcam, Cat# AB14055), rabbit polyclonal anti-pan dystrophin (1:500 Thermo, Cat# PA5-16734), mouse monoclonal anti-human DYSTROPHIN clone Dy10/12B2 (1:25 Leica Microsystems Cat# NCL-DYS3), Alexa Fluor 647-conjugated anti-human CD29 clone TS2/16 (1:50 BioLegend, Cat# 303018), rabbit monoclonal anti-CD56 (1:100 abcam Cat# ab75813), rabbit polyclonal anti-MYOD (1:200 Santa Cruz Biotechnology, Cat# sc-760), rabbit polyclonal anti-MYF5 (1:200, Santa Cruz Biotechnology, Cat# sc-302), or chicken polyclonal anti-laminin (1:1000 abcam, Cat# AB-14055, Cambridge, MA), Alexa Fluor 555-conjugated anti α-bungarotoxin (1:500 Invitrogen, Cat# B35451), rabbit polyclonal anti-synaptophysin (1:100 DAKO, Cat# A 0010) diluted in DAKO Antibody Diluent (DAKO, Cat# S0809). After PBST wash for 3 min 3 times, the following corresponding secondary antibodies were applied for 1 hr at room temperature: FITC donkey anti-mouse (1:300 Jackson Immunology, Cat# 715-095-150), Cy3 goat anti-mouse (1:500 Jackson Immunology,), Cy5 donkey anti-chicken (1:200 Jackson Immunology, Cat# 7053-175-155), Cy5 donkey anti-mouse (1:200 Jackson Immunology, Cat# 715-175-150), FITC donkey anti-rabbit (1:300 Jackson Immunology, Cat# 711-095-152). Sections were mounted with VECTASHIELD mounting medium with DAPI (H-1200 Vector Laboratories, Burlingame, CA) and all samples were examined using a Leica upright microscope.