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# Identification, isolation, and characterization of Fibro-adipogenic progenitors (FAPs) and Myogenic Progenitors (MPs) in skeletal muscle in the rat

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**TITLE:**

# Identification, Isolation, and Characterization of Fibro-adipogenic progenitors (FAPs) and Myogenic Progenitors (MPs) in Skeletal Muscle in the Rat

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**KEYWORDS:**

mesenchymal progenitors; fibro-adipogenic progenitors; myogenic progenitors; flow cytometry; fluorescence-activated cell sorting, FACS; skeletal muscle; chronic traumatic denervation; rat; muscle atrophy; fibrosis

**SUMMARY:**

This protocol outlines a method to isolate Fibro-adipogenic progenitors (FAPs) and myogenic progenitors (MPs) from rat skeletal muscle. Utilization of the rat in muscle injury models provides increased tissue availability from atrophic muscle for the analysis and a larger repertoire of validated methods to assess muscle strength and gait in free-moving animals.

**ABSTRACT:**

Fibro-adipogenic Progenitors (FAPs) are resident interstitial cells in skeletal muscle that, together with myogenic progenitors (MPs), play a key role in muscle homeostasis, injury, and repair. Current protocols for FAPs identification and isolation use flow cytometry/fluorescence-activated cell sorting (FACS) and studies evaluating their function *in vivo* to date have been undertaken exclusively in mice. The larger inherent size of the rat allows for a more comprehensive analysis of FAPs in skeletal muscle injury models, especially in severely atrophic muscle or when investigators require substantial tissue mass to conduct multiple downstream assays. The rat additionally provides a larger selection of muscle functional assays that do not require animal sedation or sacrifice, thus minimizing morbidity and animal use by enabling serial assessments. The flow cytometry/FACS protocols optimized for mice are species specific, notably

restricted by the characteristics of commercially available antibodies. They have not been optimized for separating FAPs from rat or highly fibrotic muscle. A flow cytometry/FACS protocol for the identification and isolation of FAPs and MPs from both healthy and denervated rat skeletal muscle was developed, relying on the differential expression of surface markers CD31, CD45, Sca-1, and VCAM-1. As rat-specific, flow cytometry-validated primary antibodies are severely limited, in-house conjugation of the antibody targeting Sca-1 was performed. Using this protocol, successful Sca-1 conjugation was confirmed, and flow cytometric identification of FAPs and MPs was validated by cell culture and immunostaining of FACS-isolated FAPs and MPs. Finally, we report a novel FAPs time-course in a prolonged (14 week) rat denervation model. This method provides the investigators the ability to study FAPs in a novel animal model.

**INTRODUCTION:**

Fibro-adipogenic progenitor cells (FAPs) are a population of resident multipotent progenitor cells in skeletal muscle that play a critical role in muscle homeostasis, repair, and regeneration, and conversely, also mediate pathologic responses to muscle injury. As the name suggests, FAPs were originally identified as a progenitor population with the potential to differentiate into fibroblasts and adipocytes[1] and were purported to be the key mediators of fibro-fatty infiltration of skeletal muscle in chronic injury and disease. Further study revealed that FAPs are additionally capable of osteogenesis and chondrogenesis[2–4]. Thus, they are more broadly notated in the literature as mesenchymal or stromal progenitors[3,5–8]. In acute skeletal muscle injury, FAPs indirectly aid in regenerative myogenesis by transiently proliferating to provide a favorable environment for activated muscle satellite cells and their downstream myogenic progenitor (MPs) counterparts[1,9,10]. In parallel with successful regeneration, FAPs undergo apoptosis, returning their numbers to baseline levels[1,9–11]. In contrast, in chronic muscle injury, FAPs override pro-apoptotic signals, which results in their persistence[9–11] and abnormal muscle repair.

*In vivo* studies evaluating the cellular and molecular mechanisms by which FAPs mediate muscle responses have utilized murine animal models to date[1,7,9–14]. While genetically engineered mice are powerful tools for the use in these analyses, the small size of the animal limits tissue availability for study in long-term localized injury models where muscle atrophy can be profound, such as traumatic denervation. Furthermore, measurement of muscle strength and physical function requires *ex vivo* or *in situ* measurements that necessitate termination of the mouse, or *in vivo* methods that require surgery and/or a general anaesthetic to permit evaluation of muscle contractile performance[15–20]. In rats, well validated and globally utilized muscle functional analyses, in addition to analyses for more complex motor behaviors such as gait analysis (e.g., Sciatic Function Index, CatWalk analysis) exist and are performed in awake and spontaneously moving animals[21–24]. This additionally optimizes the principles of minimal morbidity in animal experimentation, and numbers of research animals used. The rat thereby provides the FAPs investigator the added flexibility of greater injured muscle volume for protein and cellular analyses and the ability to undertake serial assessments of muscle complex static and dynamic functional activity and behaviors, in the alert animal.

FAPs have primarily been identified and isolated from whole muscle samples using flow cytometry and Fluorescence-activated cell sorting (FACS) respectively. These are laser-based

assays that are able to identify multiple specific cell populations based on characteristic features such as size, granularity, and a specific combination of cell surface or intracellular markers[25]. This is highly advantageous in the study of an organ system such as skeletal muscle, as homeostasis and regeneration are a complex, multifactorial process coordinated by a plethora of cell types. A seminal study identified FAPs, as well as MPs, using flow cytometric methods in mouse skeletal muscle[1]. They demonstrated that FAPs are mesenchymal in nature, as they lacked surface antigens specific to cells from endothelial (CD31), hematopoietic (CD45), or myogenic (Integrin-α7 [ITGA7]) origins, but expressed the mesenchymal stem cell marker Sca-1 (Stem cell antigen 1)[1] and differentiated into fibrogenic and adipogenic cells in culture. Other studies demonstrated successful isolation of mesenchymal progenitors in muscle based on the expression of an alternative stem cell marker, platelet-derived growth factor receptor alpha (PDGFRα)[2,7,8] and further analysis revealed these likely to be the same cell population as FAPs[3]. FAPs are now commonly identified in flow cytometry using either Sca-1 or PDGFRα as a positive selection marker[1,9–14,26–31]. The use of PDGFRα is preferential for human tissue, however, a direct human homologue of murine Sca-1 has yet to be identified[32]. In addition, other cell surface proteins have been reported as markers of MPs (e.g., VCAM-1), providing a potential alternative to ITGA7 as an indicator of cells of myogenic lineage during FAPs isolation[33].

While flow cytometry/FACS is a powerful methodology for studying the role and pathogenic potential of FAPs in skeletal muscle[1,9–11,13,29], it is limited technically by the specificity and optimization of its required reagents. Since flow cytometric identification and isolation of FAPs has been developed and conducted in mouse animal models[1,9–11,29], this poses challenges for researchers who wish to study FAPs in other model organisms. Many factors – such as optimal tissue size to be processed, as well as reagent and/or antibody specificity and availability – differ depending on the species used.

In addition to the technical barriers to studying FAPs in a novel animal model, they have largely been studied in an acute, toxic setting – usually via intramuscular chemical injection or cardiotoxin. Evaluation of the long-term dynamics of FAPs is limited primarily to assessment in Duchenne's muscular dystrophy, using the mdx mouse model[9–11], and models of combination muscle injury such as massive rotator cuff tear where concurrent tendon transection and denervation is performed on shoulder musculature[26–28]. The response of FAPs to the sole insult of chronic traumatic denervation, a common occurrence in work-place accidents in heavy industry, agriculture, and in birth traumas (brachial plexus injury)[34–37] with significant morbidity, has not been as well characterized, often limited to a short-term time frame[11,38].

We describe a method for identifying and isolating FAPs and MPs from healthy as well as severely atrophic and fibrotic skeletal muscle in the rat. First, identification of CD31-/CD45-/Sca-1+/VCAM-1- FAPs and CD31-/CD45-/Sca-1-/VCAM-1+ MPs using a tissue digestion and flow cytometry staining protocol is demonstrated and subsequent validation of our findings is performed through culture and immunocytochemical staining of FACS-isolated cells. Using this method, we also report a novel FAPs time-course in a long-term isolated denervation injury model in the rat.

## PROTOCOL

Investigators conducting this protocol must receive permission from their local animal ethics board/care committee. All animal work was approved by the St. Michael's Hospital Unity Health Toronto Animal Care Committee (ACC #918) and was conducted in accordance with the guidelines set forth by the Canadian Council on Animal Care (CCAC). A schematic of the flow cytometry protocol is shown in **Figure 1**. If the downstream application is FACS and subsequent cell culture, all steps should be completed with proper aseptic technique.

### 1. Muscle harvesting

1.1 Anesthetize rats using an appropriate anesthetic and sacrifice according to local vivarium and animal ethics board guidelines. This protocol harvests the gastrocnemius muscle from adult female Lewis rats (200 – 250 g), as an example. Rats were anesthetized using 2-3% Isoflurane and were sacrificed by intracardiac injection of T61.

1.2 Once the animal has been sacrificed, shave the whole hindlimb to facilitate the location of the muscle and minimize fur contamination of the harvested tissue.

1.3 Using a sterile scalpel, make two incisions in the skin: the first around the circumference of the ankle joint and the second up the midline of the medial aspect of the hindlimb from the ankle to the hip.

1.4 Peel back the skin and superficial muscle layers to reveal the underlying gastrocnemius, which originates at the medial and lateral condyles of the femur and inserts at the Achilles Tendon.

1.5 Use blunt dissection to separate the gastrocnemius from the surrounding tissue, handling the muscle only by the tendon to avoid crush injury.

1.6 Separate the gastrocnemius from its insertion by transecting the Achilles Tendon as distally as possible with sharp scissors. Once cut, grasp the Achilles tendon with forceps and gently peel the gastrocnemius off the underlaying bone. Still holding the muscle with forceps in one hand, locate the gastrocnemius' two origins and cut at the medial and lateral femoral condyles.

1.7 Blot the excised gastrocnemius gently against a sterile piece of gauze to remove as much blood as possible. Trim the muscle on a sterile surface and remove any excess connective tissue as well as the Achilles Tendon.

1.8 Place muscle in a weigh-boat and weigh using a precision scale. This protocol is optimized to digest muscle with a wet weight ranging from 200-600 mg. Operators may subdivide excess harvested tissue for other downstream assays, if desired.

1.9 Gently further divide harvested muscle to be used for flow cytometry into 3-4 smaller pieces (approximately 1-2 $cm^3$) and submerge in ice-cold 1x PBS. Keep cold on ice until all samples have been harvested.

## 2. Muscle digestion

2.1 Remove muscle from PBS and place in a sterile 10 cm cell culture dish. Gently tear and mince tissue with forceps until pieces are approximately 3-4 $mm^3$, removing as much connective tissue as possible. Once thoroughly minced, transfer to a sterile 50 mL conical tube containing 6 mL DMEM + 1% penicillin/streptomycin (P/S).

2.2 Add 10 µL of 300 mM $CaCl_2$ solution to 365 µL of Collagenase II solution (stock concentration 4800 U/mL) to activate the collagenase enzyme. Add the activated collagenase II solution to the 50 mL conical tube containing the tissue slurry. The final Collagenase II concentration is 250 U/mL.

2.3 Incubate tubes in a shaker for 1 h at 37 °C, 240 x *g*, making sure to manually swirl every 15 min to dislodge any tissue that has adhered to the side of the tube.

2.4 After 1 h, remove tubes from shaker and add the following per sample: 100 µL of Collagenase II (4,800 U/mL) and 50 µL of Dispase (4.8 U/mL).

2.5 Pipette samples using a serological pipette 15-20 times until the solution is homogenous. If processing multiple samples, use a separate sterile pipette for each sample to avoid sample cross-contamination.

2.6 Incubate again in a shaker for 30 min at 37 °C and 240 x *g*. After 15 min, shake samples by hand to dislodge adherent tissue off the side of the tube.

## 3. Generation of single cell suspension

3.1 Slowly shear samples through a 10 mL syringe with a 20 G needle for 10 cycles.

NOTE: One cycle involves taking up muscle solution into syringe, and injecting it back into tube. Ensure to minimize any bubbles by completing shearing slowly, as excessive frothing can cause additional cell death[39].

3.2 Place a 40 µm cell strainer on a sterile 50 mL conical tube and wet it by pipetting 5 mL DMEM + 10% FBS & 1% P/S.

3.3 Pipette 1 mL of the sample at a time through the cell strainer.

3.4 Wash the cell strainer by pipetting DMEM with 10% FBS and 1% P/S through the strainer to bring the total volume in the tube to 25 mL.

3.5 Split 25 mL of the sample equally into two 15 mL conical tubes and centrifuge at 15 °C, 400 x *g* for 15 min.

NOTE: Splitting the muscle solution into two 15 mL conical tubes ensures better cell recovery after centrifugation compared to a single tube.

3.6 Aspirate the supernatant and re-suspend the pellet in 1 mL 1x RBC Lysis buffer (see **Supplementary File**) at room temperature for 7 min to eliminate erythrocytes.

3.7 Bring up the volume to 10 mL with 9 mL of wash buffer (see **Supplementary File**) and spin tubes at 400 x *g*, 15 °C for 15 min.

3.8 Aspirate the supernatant and recombine pellets by re-suspending in 1 mL wash buffer.

3.9 Transfer an appropriate volume of cells to a separate 1.5 mL microcentrifuge tube and mix with trypan blue dye. Count live cells on a light microscope using a hemocytometer.

## 4. Antibody staining for flow cytometry

NOTE: The Sca-1 antibody must be conjugated to APC prior to flow cytometry/FACS experiments, as per the manufacturer's instructions. Performance must be validated for each batch of conjugates (**Figure 2**). Final conjugations can be stored in 20 µL aliquots at -20 °C and are stable for three weeks. Refer to the **Supplementary File** for full conjugation protocol.

4.1 For flow cytometry, transfer 1-2 x $10^6$ cells per experimental sample to a sterile 1.5 mL microcentrifuge tube. Bring volume up to 1 mL with wash buffer and place on ice.

4.2 For each experiment, set up the following required controls: i) unstained and ii) viability controls to accurately select for the live cell population; iii) fluorescence minus one (FMO) controls on single cell suspensions to set accurate gates for CD31-/CD45- fractions, FAPs, and MPs; and iv) single-stained compensation beads to correct for fluorescence spillover between channels.

4.2.1. For all cell controls, aliquot 5 x $10^5$ - 1 x $10^6$ cells in 1 mL of wash buffer in a 1.5 mL microcentrifuge tube and place on ice.

4.2.2. For bead controls, add 1 drop of positive compensation beads (~1.5 x $10^5$ beads per drop) to each labeled 1.5 mL microcentrifuge tube. The full complement of controls is listed in **Table 1**.

4.2.3. If the experiment is being performed for the first time, run single-stained controls for each conjugated antibody on single cell suspensions (in addition to unstained, viability, single-stained compensation bead and FMO controls) to assess the positive stained population in cells and validate staining observed on compensation beads. Validate every freshly-conjugated Sca-1::APC preparation by performing single-staining on compensation beads and single cell suspensions. Refer to **Table 1** for a full list of staining controls.

4.3 To prepare the viability control, transfer half of the volume of cells from the "viability" tube to a new 1.5 mL microcentrifuge tube. Label this tube "Dead".

4.4 Incubate "Dead" tube at 65 °C for 2-3 min to kill the cells, then place on ice. After 2-3 min, re-combine dead cells with live cells remaining in the viability control tube. This population of cells will be used to set compensation values (if needed) and properly set gates for the viability dye.

4.5 Centrifuge the single cell suspensions (experimental samples and controls) at 500 x *g*, 4 °C for 5 min.

4.6 Aspirate the supernatant and re-suspend cell pellets in 100 µL wash buffer.

4.7 Add antibodies, depending on the experimental sample or control. Refer to the staining matrix (**Table 2**) for information on antibody combinations and amounts.

4.8 Gently flick each sample to ensure complete mixing and incubate on ice in the dark for 15 min. For compensation beads, incubate at room temperature in the dark for 15 min.

4.9 For single cell suspension experimental and control samples, bring up the volume to 1 mL by adding 900 µL wash buffer. For compensation bead controls, bring up the volume to 1 mL with 900 µL of 1x PBS.

4.10 Centrifuge single cell suspension samples at 500 x *g*, 4 °C for 5 min. Centrifuge compensation bead controls at 300 x *g*, 4 °C for 5 min.

4.11 For all single cell suspension samples, aspirate and discard supernatant and re-suspend cell pellet in 300 µL wash buffer. For compensation bead controls, aspirate and discard the supernatant, re-suspend the pellet in 300 µL of 1x PBS, then add 1 drop (~1.5 x $10^5$) of negative compensation beads.

4.12 Keep all single cell suspension samples on ice under aluminum foil and proceed to flow cytometric acquisition. Compensation bead controls should also be protected from light but can be kept at room temperature.

NOTE: If experimental endpoint is FAPs identification by flow cytometry, please follow steps 5.1.1-5.1.11. If endpoint is cell isolation via FACS for culture and staining, please follow steps 5.2.1-5.2.9 and sections 6-7.

## 5. Flow cytometry and fluorescence-activated cell sorting (FACS)

### 5.1 Flow cytometry

NOTE: This protocol employs a benchtop flow cytometer equipped with 405 nm, 488 nm, and 640 nm lasers that are capable of simultaneously distinguishing 10 different colors. Bandpass

filters and their associated fluorochromes used in this protocol are as follows: 450/50 (SYTOX Blue), 530/30 (FITC), 575/25 (PE), and 670/30 (APC). Voltages for each detector are as follows: FSC 700; SSC 475; FITC 360; PE 460; PE-Cy7 600; SYTOX Blue 360; APC 570. Ensure you are trained on the proper operation of the flow cytometer or cell sorter prior to use.

5.1.1. Ensure the cytometer has been turned on for 10-20 min before use and has been primed by cleaning sequentially with clean, rinse, and sheath fluid solutions for 30-45 s each. Finish with a rinse with $dH_2O$. Ensure that an adequate volume of sheath fluid has been added to the storage container to maintain proper sample flow throughout acquisition.

5.1.2. Set up the gating strategy to identify FAPs and MPs as delineated in **Figure 3**.

NOTE: FAPs and MPs are identified by the following hierarchical gating strategy: i) SSC-A vs FSC-A (side cell scatter area versus forward cell scatter area to separate cells vs debris), ii) FSC-W vs FSC-H (forward cell scatter width versus forward cell scatter height to discriminate singlets from doublets in the FSC parameter), iii) SSC-W vs SSC-H (side cell scatter width versus side cell scatter height to discriminate singlets from doublets in the SSC parameter), iv) SSC-A vs SYTOX Blue (to distinguish live versus dead singlets), v) SSC-A vs CD31/45::FITC (to exclude CD31+ and CD45+ cells from further analysis), and vi) Sca-1::APC vs VCAM-1::PE from the CD31-/CD45- (Lineage; Lin-) population (identification of FAPs and MPs). FAPs are identified as CD31-/CD45-/Sca-1+/VCAM-1- events and MPs are identified as CD31-/CD45-/Sca-1-/VCAM-1+ events.

5.1.3. First run each single-stained compensation bead control through the cytometer on low speed to generate compensation values used to correct for any fluorescence spillover between channels. Assess compensation by comparing fluorescent signal of each control in its own detector (e.g., SSC-A vs APC for Sca-1::APC single-stained beads) as well as all other detectors. There should be two distinct populations (one with negative and one with positive signal) in the appropriate detector and only a negative population in all other detectors. Set the stopping gate to 10,000 compensation bead events and record the data.

NOTE: In between acquisition of each sample, make sure to run $dH_2O$ through the cytometer for 10-20 sec to avoid sample-to-sample contamination.

5.1.4. Next process the unstained and viability control samples to properly gate on live single cells. Set the stopping gate to 10,000 singlet events and record data.

NOTE: Approximately 5 min before acquisition of each single cell suspension sample with the exception of the unstained sample, add 1 µL of SYTOX Blue viability dye (300 µM working concentration diluted from 1 mM stock solution) to each sample and flick gently to mix (final concentration 1 µM).

5.1.5. Then acquire the remaining single cell suspension control samples. Assess each FMO control with its appropriate plot in the gating strategy (**Figure 3**). For example, assess the FITC signal of the CD31+CD45 FMO to ensure an accurate CD31-/CD45- gate. An optimal example is

shown in **Figure 3G**. If the protocol is being performed for the first time, single-stained controls on cells should be run before the acquisition of FMO controls.

5.1.6. Assess the fluorescent signal of each single-stained cell sample in its appropriate detector as well as in all other detectors to validate proper compensation. Set the stopping gate to 10,000 live singlet events and record on the software.

5.1.7. Once all controls (single cell suspensions and beads) have been processed, prepare all experimental samples by first measuring and recording the volume of each sample. These measurements will be used to accurately quantify FAPs and MPs, as described in step 5.1.11. Then, add 50 µL of precision counting beads and gently mix by pipetting up and down 2-3 times.

5.1.8. Briefly run the first experimental sample to validate identification of the counting bead population. This population appears as a small distinct cluster separate from the general cell population on the FSC-A vs SSC-A plot (**Figure 3A**, red box). Create a gate around the counting bead population. Then acquire data for each experimental sample by processing through the cytometer on low speed. Set the stopping gate to 10,000 counting bead events and record.

NOTE: Investigators may alternatively identify counting beads by setting up an additional plot assessing SSC-A versus any of the detectors, as the counting beads are fluorescent in all detectors.

5.1.9. After all samples have been processed, clean the cytometer using the appropriate protocols. Export all data for analysis.

5.1.10. Open all data files on an appropriate flow cytometry analysis software. Set the gating strategy as used for data acquisition as described in step 5.1.2. Examine controls in the same order as in data acquisition (e.g., unstained, viability, single stain, then FMO controls) to re-validate the gating strategy. Once accurate gates have been set using FMO controls, apply the gates to all experimental samples. Export raw data as a spreadsheet for quantification.

5.1.11. Calculate the number of FAPs and MPs in each experimental sample using the counting beads:

$$\text{Absolute Count}\left(\frac{\text{cells}}{\mu\text{L}}\right) = \left(\frac{[\text{Acquired Cell Count (\#cells) x Counting Beads Volume (µL)}]}{[\text{Acquired Bead Count (\#beads) x Sample Volume (µL)}]}\right) \text{x Bead conc (\#beads/uL)}$$

where, Acquired Cell Count is the number of recorded events of pertinent cell population (e.g. FAPs or MPs) on the acquisition software; Acquired Bead Count is the number of recorded events of counting beads on the acquisition software; Counting Beads Volume is the volume of counting bead solution added in step 5.1.7; Sample Volume is the volume of each stained experimental sample prior to addition of counting beads.; Bead Concentration is the number of beads per µL solution; this value is found on the product datasheet.

## 5.2. FACS - sorting for cell culture

NOTE: This protocol performs FACS on a cell sorter equipped with 4 lasers (UV, Violet, Blue, Red) that is capable of simultaneously distinguishing 11-14 colors. The experimental sample staining (section 4) and flow cytometry protocol are followed, with the exceptions delineated below, to optimize the FACS workflow:

5.2.1. Increase the concentration of cells in the experimental samples to be sorted to 7 x $10^6$ cells/mL to generate robust yields of FAPs and MPs.

5.2.2. To account for this significant increase in the cell concentration, double all antibody concentrations in the experimental samples to be sorted.

5.2.3. Process the final stained cell samples through a 40 µM cell strainer cap affixed to a 5 mL polystyrene tube immediately prior to sorting to reduce cell clumping and increase sort yields.

5.2.4. Collect single, live rat FAPs and MPs directly from the cell sorter into a 5 mL polypropylene collection tube containing 1 mL of sterile, 100% Fetal Bovine Serum (FBS). Keep cells on ice until sorting is complete.

NOTE: If conducting FACS at an off-site location, transfer all sorted cells on ice and in a secured, covered container.

5.2.5. Working in a sterile biosafety cabinet (BSC), bring volume of sorted cells up to 7 mL with appropriate growth media (e.g., FAP growth media (FAP GM) for sorted FAPs, and MP growth media (MP GM) for sorted MPs; see Supplementary File for recipes) and centrifuge at 500 x g, 4 °C for 7 min to remove as much residual wash buffer as possible.

5.2.6. Resuspend pellets in 1 mL of appropriate growth media and plate into a 12-well plate containing a sterile, collagen-coated 12 mm glass coverslip/well for subsequent immunostaining (see section 6).

NOTE: If immunocytochemistry staining for collagen, plate sorted cells into a 12 well plate containing a sterile, laminin-coated 12 mm glass coverslip/well, instead of collagen-coated. If immunocytochemistry experiments of immediately isolated progenitors are required, seed FAPs and MPs at a density of 15,000 cells per $cm^2$ and proceed directly to step 6.1. For long-term cultures to induce progenitor differentiation, seed FAPs at a density of 5,000 cells per $cm^2$, and MPs at a density of 7,500 cells per $cm^2$.

5.2.7. Incubate cells at 37 °C and 5% $CO_2$ in a cell culture incubator. After 72 h in culture, change half of the media. Change media fully every 2-4 d after.

5.2.8. To induce myocyte development, switch MPs cultures to MP differentiation (MD) medium on Day 9 of culture. To induce adipocytes, switch FAPs cultures to FAP adipogenic differentiation (AD) medium on Day 10 of culture.

5.2.9. To induce fibrogenesis, FAPs may be switched to fibrogenic differentiation (FD) media at variable times during culture, or alternatively, may be seeded directly into FD media following isolation (step 5.14) (See **Supplementary File** for all media recipes).

## *6.* Immunocytochemistry of cultured FAPs and MPs

6.1 To validate cell sorting and demonstrate purity of FAPs and MPs cultures, immunostain with cell-type specific markers including PDGFRα (FAPs marker), Pax-7 (muscle stem [satellite] cell marker), Fibroblast-specific protein (FSP-1, fibroblast marker), Perilipin-1 (Plin-1, adipocyte marker), Collagen type 1 (Col1a1, indicator of fibrosis), Myosin Heavy Chain (MHC, mature myocyte marker).

6.1.1. For immunostaining of freshly sorted cells, centrifuge the 12-well plate at 200 x g for 3 min at room temperature to facilitate adherence of cells to the coverslip/well. This step is not necessary for long-term cultures. Remove culture media.

6.1.2. For immunostaining with FSP-1, fix cells with 1 mL 100% methanol (MeOH) for 2 min at 4 °C. If immunostaining for PDGFRα, Plin-1, Pax7 or Col1a1, fix cell cultures with 1 mL 4% PFA in 1x PBS for 15 min at room temperature. MHC immunostaining tolerates either fixative.

NOTE: For methanol-fixed cells, skip step 6.2 and proceed to step 6.3.

6.2 Aspirate 4% PFA and quickly wash cell cultures 3-4 times with 1x PBS. Add 1 mL of 100 mM Glycine in 1x PBS and incubate for 10 min at room temperature to inactivate residual PFA. Aspirate and wash 1-2 times with 1x PBS.

NOTE: Cells can be left at this stage in 1 mL 1x of PBS, wrapped in cling film and stored at 4 °C for 7-10 days maximum.

6.3 After washing, add 1 mL of 0.1% Triton-X in 1x PBS and incubate for 20 min to permeabilize cell membranes.

6.4 Wash wells 2-3 times with 1-2 mL of 1x PBS then block cells with 1 mL of 1x PBS + 3% BSA per well for 1 h at room temperature.

6.5 Pipette 80 µL of primary antibody diluted in 1x PBS + 3% BSA (PDGFRα 1:100, Pax7 neat, FSP-1 1:50, Plin-1 1:400, Col1a1 1:250, MHC 3 µg/mL) onto a piece of parafilm taped to a mobile container. Using sterile fine forceps, carefully lift the coverslip out of the well and invert onto the

drop of antibody solution. Incubate coverslip with two wet pieces of paper towel and cover container in plastic film to avoid evaporation of the antibody solution. Incubate overnight at 4 °C.

NOTE: Staining coverslips out of the well utilizes less antibody (~80 µL) than staining inside the well (500 µL minimum).

6.6 On Day 2, leave coverslips at room temperature for 30 min to warm. Using forceps carefully right and transfer coverslips back to their respective wells (cells facing up) and wash 2-3 times with 1-2 mL of 1x PBS for 2 min each to remove as much primary antibody as possible.

6.7 Using the same staining technique as with primary antibody staining, stain cells with goat anti-rabbit Alexa Fluor 488 secondary antibody (1:400) to detect FSP-1, Plin-1, Col1a1, or PDGFRα and goat anti-mouse Alexa Fluor 555 secondary antibody (1:300) to detect MHC or Pax-7. Incubate cells for 1 h at room temperature and keep cells protected from light.

6.8 Return cells to the well and incubate cells with Hoechst (1:10,000) for 2-4 min at room temperature. Wash cells another 2-3 times with 1x PBS for 2 min each to remove excess Hoechst.

6.9 Mount coverslips onto glass slides using an anti-fade fluorescent mounting medium and leave slides to dry overnight in the dark at room temperature. Store mounted coverslips at 4 °C in the dark.

**7. Oil Red O (ORO) staining of cultured FAPs and MPs**

7.1 Perform ORO staining on non-permeabilized cells, as permeabilization of the cell membrane can result in non-specific/undesired staining of non-adipogenic cell types. Prior to commencing staining, prepare an ORO working stock (See **Supplementary File** for the recipe) and incubate at room temperature for 20 min.

7.2 After 20 min, filter the solution using a 0.2 µm filter in order to remove any undissolved aggregates.

7.3 Aspirate media from well and add 1 mL of 10% Neutral Buffered Formalin (10% NBF). Incubate for 5 min at room temperature.

NOTE: Cell confluency can result in lifting from the well/coverslip. Take care when aspirating/adding solutions.

7.4 Aspirate and add 1 mL of fresh 10% NBF and incubate for at least 1 h at room temperature.

NOTE: The protocol can be stopped at this point, as cells can be left in 10% NBF overnight.

7.5 Quickly wash the wells once with 1 mL of 60% isopropanol, then aspirate and allow the wells to dry completely (approximately 2 min).

7.6 Add 400 µL Oil Red O working stock per well and incubate for 10 min at room temperature, making sure to avoid pipetting any ORO on the walls of the plate.

7.7 Remove all of the Oil Red O and quickly wash the well 4 times with $dH_2O$.

NOTE: If stained wells contain coverslips, mount using the same technique as described in step 6.9.

7.8 Image either mounted coverslips or the stained well using a brightfield microscope.

**8. Tissue staining of contralateral and denervated rat gastrocnemius sections**

**8.1. Picrosirius Red (PSR)**

8.1.1. Perform PSR staining on 5 µm-thick, formalin-fixed paraffin embedded (FFPE) rat gastrocnemius histologic sections as previously described[40].

**8.2. Oil Red O (ORO)**

8.2.1. Fix 5 µm-thick isopentane-frozen rat gastrocnemius histologic sections in 4% PFA for 10 min, incubate in 60% isopropyl alcohol for 1 min.

8.2.2. Stain with ORO working stock for 12 min. Incubate in 60% isopropyl alcohol for 1 min, wash for 10 min in $dH_2O$. Mount on coverslips using a water-soluble mounting media.

**8.3. Sca-1 and laminin tissue fluorescent immunohistochemistry (IHC)**

8.3.1. Perform fluorescent IHC is performed on 5 µm-thick isopentane-frozen rat gastrocnemius histologic sections.

8.3.2. Hydrate samples in 1x PBS for 5 min, fix in 4% PFA for 10 min then incubate samples in tissue IF blocking solution (see **Supplementary File**) for 90 min.

8.3.3. Incubate with anti-Sca-1 primary antibody (1:500) diluted in 1x PBS + 0.05% Tween at 4 °C overnight.

8.3.4. On Day 2, wash three times in 1x PBS + 0.05% Tween for 5 min each, then incubate in goat anti-rabbit Alexa Fluor 555 (1:500) for 1 h.

8.3.5. Wash again (as before), incubate with blocking solution for 1 h, then add anti-laminin primary antibody (1:500) diluted in 1x PBS + 0.05% Tween for 1 h.

8.3.6. Wash again (as before), then incubate in goat anti-rabbit Alexa Fluor 488 (1:500) for 1 h (for laminin).

8.3.7. Wash again (as before) then incubate in DAPI (1:10,000) for 4 min. Wash and mount on coverslips using anti-fade mounting medium.

**REPRESENTATIVE RESULTS:**

**Identifying FAPs and MPs via flow cytometry using a novel antibody panel including Sca-1 and VCAM-1**

The gating strategy for identifying FAPs in rat muscle is based upon flow cytometry protocols in the mouse[29], which gate on CD31 (endothelial) and CD45 (hematopoietic) positive cells (termed the lineage [Lin]) and examines the fluorescent profile of FAPs marker Sca-1 and MPs marker ITGA7 from the linage-negative (Lin-) population. In the absence of commercially available, fluorophore-conjugated, and flow cytometry-validated antibodies for rat Sca-1 and ITGA7, we self-conjugated a rat Sca-1::APC antibody, and undertook an alternative strategy to identify the MPs. As VCAM-1 was recently shown to be an efficient single positive selection marker for the isolation of rat MPs[33] and a rat-specific, conjugated, flow cytometry-validated antibody targeting VCAM-1 is available, we utilized VCAM-1 to identify MPs, as opposed to ITGA7.

We confirmed successful conjugation and performance of the Sca-1:APC antibody with single staining of compensation beads and cell suspensions generated from healthy rat gastrocnemius (**Figure 2A,B**). A five-point titration of Sca-1::APC (**Figure 2C**) was performed in addition to all other antibodies (CD31::FITC, CD45::FITC, VCAM-1:PE) used in the protocol (**Supplementary Figure 1**), to identify the optimum concentrations. Using this novel panel design, putative FAPs and MPs were simultaneously identified from healthy gastrocnemius (**Figure 3**), whereby cells single-positive for Sca-1 (Lin-/Sca-1+/VCAM-1-) were designated FAPs (red box) and cells single-positive for VCAM-1 (Lin-/Sca-1-/VCAM-1+) were designated MPs (blue box). We also identified a population of cells double positive for Sca-1 and VCAM-1 (Lin-/Sca-1+/VCAM-1+) (**Figure 3F**; upper right quadrant).

**Validation of identification of FAPs and MPs by FACS and cell culture**

To validate the protocol presented for flow cytometric identification of FAPs and MPs in rat skeletal muscle, we sought to isolate live cells for culture *in vitro*. Using FACS, viable FAPs and MPs were isolated using the same gating strategy as in the flow cytometric analysis. Approximately 20,000-40,000 FAPs and 30,000-50,000 MPs were collected for cell culture from a single gastrocnemius muscle from a 230 g rat.

To confirm the purity of each population, we co-immunostained freshly sorted FAPs and MPs for PDGFRα and Pax7. PDGFRα is the second mesenchymal progenitor marker, besides Sca-1, commonly used to identify FAPs[2,7,8]. Pax-7 is a well-recognized and widely used marker of muscle satellite (stem) cells[41]. The sorted population of FAPs displayed positive staining for PDGFRα with no contamination by Pax7 positive cells (**Figure 4A**; top row). Conversely, the sorted population of MPs stained positive for Pax7 with an absence of PDGFRα positive cells (**Figure 4A**; bottom

row), validating the ability of Lin-/Sca-1+/VCAM-1- and Lin-/Sca-1-/VCAM-1+ cell surface antigen profiles to isolate pure populations of FAPs and MPs respectively.

We next cultured sorted FAPs and MPs over a time course of 10-12 days in conditions to induce adipogenic, fibrogenic, and myogenic differentiation. By day 12, FAP cultures subjected to adipogenic conditions contained cells with either a fibroblast-like morphology or a multilocular morphology similar to that of white pre-adipocytes with fat droplets (data not shown). Immunostaining for fibroblast specific protein-1 (FSP-1) confirmed fibroblast differentiation, while staining for Plin-1 (Perilipin-1; an adipocyte marker)[42] and Oil red O confirmed the differentiation of adipocytes and the presence of neutral triglycerides and lipids, respectively (**Figure 4B**). The absence of contaminating cells from a myogenic lineage in the FAPs cultures was confirmed by co-immunostaining for myosin heavy chain (MHC), a marker of differentiated myocytes. FAP cultures subjected to fibrogenic differentiation (FD) were assessed for Collagen type 1 (Col1a1) expression, one of the main FAPs-derived collagens[8]. Co-immunostaining for Col1a1 and MHC at Day 11 revealed robust Collagen type 1 expression with no contaminating MHC positive cells (**Figure 4B**). Final confirmation of purity of the FAPs population was undertaken by culturing FAPs in myogenic media, to encourage the outgrowth of any contaminating MPs. No MHC positive cells were observed (**Supplementary Figure 2A**).

MP cultures subjected to myogenic conditions demonstrated MHC-expressing mature myocytes and multi-nucleated myotubes 12 days post plating (**Figure 4C**). Co-immunostaining of the MP cultures with FSP-1, and Plin-1 demonstrated the absence of contaminating fibroblasts or adipocytes, respectively. Similarly, ORO staining did not demonstrate lipid contamination (Fig. 4C). Col1a1, which is highly expressed by fibroblasts, has also been reported to be produced to a lesser extent by myogenic precursors and myoblasts, although there are contradictory data in the literature in this regard[8,43,44]. While co-immunostaining of MPs with Col1a1 and MHC revealed myocyte differentiation of our MP culture, no evidence of Col1a1 immunopositivity was found (**Figure 4C**). To further confirm the purity of the population, MP cultures were subjected to adipogenic or fibrogenic conditions to encourage the growth of adipocytes and fibroblasts (**Supplementary Figure 2B**). No contaminating cells from the FAPs lineage were observed.

While we had demonstrated the ability to identify and sort pure populations of FAPs and MPs from rat muscle, we next sought to determine the identity of the Lin-/Sca-1+/VCAM-1+ (double positive) cells. Since Sca-1 expression has been reported on a very small proportion of MPs (approximately 3%) in healthy muscle[45] and similarly few FAPs (approx. 4%) have been reported to express VCAM-1 in healthy muscle[10], immunostaining of freshly sorted Lin-/Sca-1+/VCAM-1+ cells was performed for PDGFRα and Pax7 along with culturing them in myogenic, adipogenic, and fibrogenic conditions to induce myogenesis, adipogenesis and fibrogenesis respectively. It was found that the double-positive cells were a mixed population of MPs and FAPs, with freshly sorted cells immunostaining positive for either PDGFRα or Pax7 (**Supplementary Figure 3A**) and cultured cells were differentiating into mature myocytes, adipocytes or fibroblasts (**Supplementary Figure 3B,C**).

**ITGA7 vs VCAM-1 to identify MPs during flow cytometric FAPs identification**

While a successful protocol for flow cytometric identification of rat FAPs and MPs using Sca-1 and VCAM-1 respectively was established, we sought to determine if a self-conjugated ITGA7 antibody could similarly be used to identify MPs instead of VCAM-1, as is standard in the mouse. A rat-specific ITGA7 antibody to PE-Cy7 (see **Supplementary File**) was conjugated and the antibody's performance was validated on commercial compensation beads and single cell suspensions were generated from rat gastrocnemius (**Supplementary Figure 4A-C**). The ITGA7::PE-Cy7 antibody performed adequately on single staining and FMO experiments (**Supplementary Figure 4D**). However, when full staining gastrocnemius cell suspensions with CD31::FITC, CD45::FITC, Sca-1::APC, and ITGA7::PE-Cy7, an interaction became evident between the Sca-1::APC and ITGA7::PE-Cy-7 antibodies. Subsequent culture of both the FACS sorted Lin-/Sca-1+/ITGA7- cells (purported FAPs) and Lin-/Sca-1-/IGTA7+ cells (purported MPs) (**Supplementary Figure 4E**) yielded predominantly FAPs and with few MPs (**Supplementary Figure 4F**), indicating the interaction between Sca-1::APC and ITGA7::PE-Cy-7 antibodies negatively impacted the specificity of cell identification.

**A novel FAPs time-course in long-term denervated skeletal muscle**

As FAPs dynamics have been assessed in the context of short-term traumatic denervation, in murine models[11, 38], we sought to validate the performance of our method for FAPs isolation from both healthy and severely atrophic, fibrotic muscle. Rats were subjected to traumatic long-term denervation injury using the well-validated unilateral tibial nerve transection model[46] with gastrocnemius muscle harvested at four serial timepoints over 14 weeks post-denervation from the denervated limb and contralateral innervated limb (to serve as an internal control). As has been previously reported, denervated muscle demonstrated progressive atrophy (**Figure 5A,B**), with increasing fibrosis and fat deposition (**Figure 5C-F**) over time[47].

Our protocol generated an adequate number of cells for flow cytometric analysis, even though 12 and 14 week-denervated gastrocnemius weighed approximately 0.2-0.3 g (15-20% of respective contralateral control muscle) (**Figure 5B**). We observed up-regulation of FAPs in the denervated gastrocnemius muscle compared to the innervated contralateral control muscle, maintained for the 14 week duration of the experiment (**Figure 6A,B**), concordant with the progressive muscle fibrosis and fat deposition. Sca-1 immunostaining of muscle histologic cross sections confirmed localization of Sca-1 expressing cells to areas of fibro-fatty change in 14 week-denervated muscle (**Figure 5G**), in addition to baseline expression in the interstitium between myofibers in healthy muscle. A distinct subset of cells emerged in the FAPs population over time post-denervation characterized by a robust increase in Sca-1 signal (Sca-1 high; **Figure 6A**, red box) on flow cytometric analysis, compared to the FAPs basal Sca-1 expression (Sca-1 Med/Low; Fig. 6A, green box). Quantification of these subpopulations revealed differential dynamics over time; Sca-1 High FAPs increased significantly at 12- and 14-weeks post-denervation (**Figure 6B**), comprising approximately half of the total FAPs population (**Figure 6C**). In contrast, Sca-1 Med/Low FAPs were the dominant subpopulation at 2- and 5-weeks post-denervation (**Figure 6C**) and showed only a transient increase in levels early post-denervation (**Figure 6B**).

In contrast to the sustained presence of FAPs in long-term denervated muscle, MPs demonstrated an expected biphasic response to denervation. Initially the MPs increased in

denervated muscle above that seen in the control limb, but at 5 weeks post-denervation the population began to decline (**Figure 6D**). In keeping with the well-known exhaustion of the muscle satellite cell population in long-term denervated muscle[47], by 12 weeks MPs were either at or below baseline levels post tibial nerve transection.

We also analyzed the dynamics of the Lin-/Sca-1+/VCAM-1+ population (**Supplementary Figure 3D-E**). While the frequency of double positive cells relative to the Lin- population progressively decreased in denervated muscle over time, when the absolute cell count was determined per gram of muscle a temporary increase in the double positive population was observed, prior to falling to or below baseline levels by 14 weeks post-denervation.

**FIGURE LEGENDS:**

**Figure 1: Graphical schematic for FAPs and MPs identification, isolation, and culture:** Graphical schematic depicting FAPs and MPs identification from rat gastrocnemius. Samples are harvested and mechanically minced before undergoing two sequential enzymatic digestions. Tissue preparations are then processed and filtered to generate a single cell suspension. A cocktail of fluorescently-conjugated antibodies is added to each sample which is then run through a flow cytometer or cell sorter to respectively identify or isolate FAPs and MPs.

**Figure 2: Sca-1::APC self-conjugated antibody validation and titration.** Validation of Sca-1::APC antibody conjugation by single-staining on commercial compensation beads (**A**) and single cell suspensions generated from healthy rat gastrocnemius muscle (**B**). Distinct populations of both Sca-1 labeled beads and cells are evident (black boxes). Antibody titration was performed by testing five different concentrations of Sca-1::APC on single cell suspensions (**C**); the optimal concentration was chosen based on greatest fluorescence intensity with minimal background staining and found to be batch dependent. A representative titration is shown with the batch specific optimal concentration indicated by the red box.

**Figure 3: Flow cytometry identification of FAPs and MPs in rat gastrocnemius.** (**A-F**) Gating strategy for flow cytometric analysis of FAPs and MPs. (**A**) Samples are first gated to exclude debris (black box) as well as to gate for counting beads (red box). Cells are then gated to exclude doublets both by front scatter (FSC) (**B**) and side scatter (SSC) characteristics (**C**). The viability of resulting single cells is assessed by staining with SYTOX Blue (**D**). SYTOX Blue negative (live) singlets are then assessed for FITC signal identifying CD31 and CD45 (Lineage; Lin) in order to exclude Lin+ fractions from further analysis (black box Lin- cells) (**E**). The Lin- population is then assessed for Sca-1::APC versus VCAM-1::PE signal (**F**). FAPs are identified as Lin-/Sca-1+/VCAM-1- (F; red box) while MPs are identified as Lin-/Sca-1-/VCAM-1+ events (F; blue box). A Lin-/Sca-1+/VCAM-1+ double positive population is also evident (upper right quadrant). (**G**) FMO (Fluorescence Minus One) controls for CD31::FITC and CD45::FITC demonstrate proper compensation and gating for Lin- cells. (**H-I**) Sca-1::APC versus VCAM-1::PE plots of FMO controls demonstrate proper compensation and gating for FAPs (Sca-1 FMO) and MPs (VCAM-1 FMO).

**Figure 4: Sorted FAPs and MPs cell culture and immunostaining.** (**A**) Co-immunostaining for PDGFRα (green) and Pax7 (red) on freshly sorted FAPs (top row) and MPs (bottom row). Nuclei

stain blue with DAPI. FAPs solely express PDGFRα and no Pax7 positive cells are evident, while MPs exclusively express Pax7 with no PDGFRα positive cell contamination, demonstrating the purity of both sorted populations. (**B**) FAPs at Day 12 in adipogenic differentiation media (AD) stain positive for Fibroblast-specific Protein 1 (FSP-1; green) and Perilipin-1 (Plin-1; green), demonstrating differentiated fibroblasts and adipocytes, respectively. Nuclei are stained with DAPI (blue). Oil Red O (ORO) staining (red) indicates the presence of neutral triglycerides and lipids arising from mature adipocytes by Day 12. FAPs subjected to fibrogenic differentiation media (FD) demonstrate expression of FAPs-derived Collagen type 1 (Col1a1; green). FAPs cultured in either adipogenic or fibrogenic media do not co-immunostain for myosin heavy chain (MHC, red), indicating an absence of contaminating myocytes. (**C**) MPs grown in myogenic differentiation media (MD) on Day 12 display MHC positive (red) staining demonstrating the presence of mature myocytes and fused multinucleated myotubes. Nuclei are stained with DAPI (blue). MP cultures were clear of fibroblast and adipocyte contamination as indicated by the absence of co-immunostaining for FSP-1 (green), Plin-1 (green) and ORO staining. MPs grown on laminin to accommodate Co1a1 immunostaining do not display the same degree of myotube fusion by Day 12 as occurs on collagen. Col1a1 (green) is absent from MP cultures.

**Figure 5: Atrophy, fibrosis, and fatty infiltration of long-term denervated muscle.** (**A**) Representative harvested gastrocnemius muscles at four serial timepoints post-denervation. Gastrocnemius denoted CONTRA is a representative contralateral limb sample from a two-week denervated animal. (**B**) Quantification of muscle atrophy post-denervation expressed as the ratio of denervated (DEN) gastrocnemius wet weight to that of the contralateral (CONTRA) limb. (**C**) Picrosirius Red (PSR) stained histologic cross-sections of gastrocnemius harvested 2 or 14 weeks post-denervation demonstrate progressive fibrosis. Muscle stains yellow, collagen stains red. (**D**) Fibrosis quantified by determining area of PSR positive tissue relative to total tissue area (**E**) Oil Red O (ORO) stained cross-sections of gastrocnemius harvested 2- or 14-weeks post-denervation, counter-stained with hematoxylin. Lipids stain red. (**F**) Quantification of fatty infiltration quantified by determining area ORO stained tissue relative to total tissue area. (**G**) Gastrocnemius histologic cross-sections harvested 2- or 14-weeks post-denervation, immunostained for laminin (green) and Sca-1 (red). Laminin positively stains the basal lamina that surrounds individual myofibers. Sca-1 identifies multiple progenitor cell types. Nuclei are stained with DAPI (blue). Inset shows localization of Sca-1+ cells outside the basal lamina in healthy muscle; yellow arrows denote presence of Sca-1+ cells within fibrotic areas. (Data are mean +/- S.D; n=3 for 2 and 14 week timepoints. Data in panel B analyzed by one-way ANOVA, data in panels D and F analyzed by two-way ANOVA. Post-hoc Sidak's test performed to correct for multiple comparisons. * = $P<0.05$ between CONTRA and DEN; # = $P<0.05$ between samples)

**Figure 6: FAPs and MPs dynamics in long-term denervated rat gastrocnemius.** (**A**) Representative Sca-1::APC versus VCAM-1::PE panels of the Lin- (CD31-/CD45-) population from muscle samples harvested 2-, 5-, 12-, or 14-weeks post-denervation. Top row shows plots from the denervated (DEN) gastrocnemius, while the bottom row shows those from the contralateral, unoperated (CONTRA) gastrocnemius. Lin-/Sca-1+/VCAM-1- FAPs (Total) are subdivided into Sca-1 High (red box) and Sca-1 Med/Low (green box) fractions, while Lin-/Sca-1-/VCAM-1+ MPs are shown in the blue box. (**B**) Quantification of FAPs (Total, Sca-1 High, Sca-1 Med/Low) at each

timepoint post-denervation, reported as the frequency (%) of Lin- cells (top row) and the number of cells per gram muscle (bottom row) normalized to the contralateral control gastrocnemius. (**C**) Relative proportions of Sca-1 High and Sca-1 Med/Low subpopulations of the total FAPs population. (**D**) Quantification of MPs at each timepoint post-denervation reported as the frequency (%) of Lin- cells (left graph) and as the number of cells per gram muscle (right graph) normalized to the contralateral control. (Data are mean +/- S.D; n=4 for 2 and 12 week timepoints; n=5 for 5 week timepoint; n=2 for 14 week timepoint. Data analyzed by one-way ANOVA; Post-hoc Sidak's test to correct for multiple comparisons. # = $P<0.05$.)

**Supplementary Figure 1: Antibody validation and titration.** Validation of CD31::FITC (A-C), CD45::FITC (D-F), and VCAM-1::PE (G-I) commercially-conjugated antibodies on compensation beads (A,D,G) and single-cell suspensions from healthy rat gastrocnemius muscle (B,E,H). Each antibody was titrated at 5 different concentrations (C,F,I). The optimal concentration was chosen based on greatest fluorescence intensity with minimal background staining (red boxes).

**Supplementary Figure 2: Sorted FAPs and MPs in reverse differentiation culture conditions.** (**A**) FAPs cultured in myogenic differentiation media (MD) at Day 12 co-immunostained for FSP-1 (green), Plin-1 (green) or Col1a1 (green) and MHC (red) do not demonstrate myocyte contamination. Positive FSP-1, and Col1a1 staining reveal FAPs differentiation to fibroblasts. ORO staining (red) reveals lipid production although Plin-1 positive adipocytes are not readily visible. Nuclei stained with DAPI (blue). (**B**) MPs subjected to adipogenic differentiation media died. MPs cultured in FAP Growth Media (FAP GM) for 12 days and co-immunostained for FSP-1 (green) or Plin-1 (green) and MHC (red) demonstrate differentiated myocytes and myotubes with an absence of FSP-1 or Plin-1 positive cells. The absence of ORO staining further confirms the lack of adipogenic cells in culture. MPs grown in fibrogenic differentiation media (FD) and co-immunostained for Col1a1 and MHC show mature myocytes and an absence of Col1a1 positive cells. MPs grown on laminin to accommodate Col1a1 immunostaining do not display the same degree of fusion to myotubes at 12 days in culture as occurs on collagen.

**Supplementary Figure 3: Lin-/Sca-1+/VCAM-1+ cells are a mixed population of FAPs and MPs.** (**A**) PDGFRα and Pax7 co-immunostaining of freshly isolated Lin-/Sca-1+/VCAM-1+ cells show a mixed population of PDGFRα single positive (white arrows) and Pax7 single positive (yellow arrow) cells identifying FAPs and MPs, respectively. (**B**) Lin-/Sca-1+/VCAM-1+ cultures on Day 12 grown in myogenic differentiation media (MD) contain both FSP-1 single positive (green) and MHC single positive (red) cells identifying fibroblasts and myocytes/tubes, respectively. An absence of Plin-1 (green) and ORO (red) staining indicate the absence of mature adipocytes. Co-immunostaining for Col1a1 (green) and MHC (red) show mature myocytes and fibroblasts. (**C**) Lin-/Sca-1+/VCAM-1+ cells on Day 12 grown in adipogenic differentiation media (AD) similarly show a mix of FSP-1 (green) single positive and MHC (red) single positive cells, but also with Plin-1 (green) single positive cells and ORO staining present, confirming the differentiation of fibroblasts, myocytes and adipocytes. Cultures grown in fibrogenic differentiation media (FD) show mature myocytes and Col1a1 single positive cells (fibroblasts). (**D**) Representative Sca-1::APC versus VCAM-1::PE panels of the Lin- (CD31-/CD45-) population from denervated muscle samples harvested 2-, 5-, 12-, or 14-weeks post-denervation; Lin-/Sca-1+/VCAM-1+ population

are indicated in the purple box. (**E**) Quantification of Lin-/Sca-1+/VCAM-1+ cells across four serial time-points post-denervation, reported as the frequency of Lin- cells (left graph) and cells per gram muscle (right graph) normalized to the contralateral gastrocnemius. (Data are mean +/- S.D; n=4 for 2 and 12 week timepoints; n=5 for 5 week timepoint; n=2 for 14 week timepoint. Data were analyzed by one-way ANOVA; post-hoc Sidak's test to correct for multiple comparisons; # = $P<0.05$.)

**Supplementary Figure 4: ITGA7 as a marker of myogenic cells in flow cytometry of rat skeletal muscle.** Validation of ITGA7::PE-Cy7 antibody conjugation by single-staining on commercial compensation beads (**A**) and single cell suspensions generated from healthy rat gastrocnemius muscle (**B**). Populations of both ITGA7 labeled beads and cells are evident (black boxes) (**C**) Antibody titration was performed by testing five different concentrations on single cell suspensions. Red box indicates ideal concentration. (**D**) Plots of Fluorescence Minus One (FMO) controls demonstrate the absence of fluorescence spillover across the antibody conjugates, but full stain of cell suspensions reveals a non-specific interaction between Sca-1::APC and ITGA7::PE-Cy7 (green box). Gating (**E**) to separate Lin-/Sca-1+/ITGA7- cells (purported "FAPs") and Lin-/Sca-1-/IGTA7+ cells (purported "MPs"). (**F**) Co-immunostaining of FACS-sorted cell cultures at 12 days post plating with Perilipin-1 (green) and MHC (red) demonstrates both groups of sorted cells matured predominantly into adipocytes with few myocytes present.

**Table 1: Flow cytometry antibody staining controls.** Full complement of antibody staining controls. If the experiment is being performed for the first time, single stained controls should be run on both compensation beads and single cell suspensions. For all subsequent experiments, single stained controls only need to be run on compensation beads.

**Table 2: Flow cytometry antibody staining matrix.** The antibody amount for staining of experimental and control samples for flow cytometry is shown. All staining is performed in a total volume of 100 µL of wash buffer. *The optimal amount of self-conjugated Sca-1::APC antibody may vary depending on batch used. All freshly conjugated Sca-1::APC batches should be first validated by single-staining both compensation beads and single cell suspensions.

**Supplementary file: Reagent recipes.**

**DISCUSSION:**

An optimized, validated FAPs isolation protocol for rat muscle is essential for researchers who wish to study injury models that are not feasible in the mouse for biologic or technical reasons. For example, mice are not an optimal animal model in which to study chronic local, or neurodegenerative injuries such as long-term denervation. Biologically, the short lifespan and rapid aging of mice make it difficult to accurately delineate the muscle sequalae due to denervation from the confounding factor of aging. From a technical standpoint, the drastically reduced muscle mass due to severe atrophy would be insufficient for effective flow cytometric analysis. In fact, mouse FAPs isolation protocols suggest grouping healthy muscles together to better increase yield of sorted FAPs[29]. While this solves the technical barrier of obtaining adequate muscle tissue for analysis, it simultaneously limits researchers from assessing FAPs on

a muscle-specific level. Since specific muscle groups vary inherently in their fiber type composition, degree of vascularization, and mitochondrial numbers[47] for example, combining multiple different muscles for flow cytometric analysis prevents muscle-specific FAPs characterization. In contrast, we show that both healthy and long-term denervated, atrophic and fibrotic rat gastrocnemius provide an adequate amount of starting material for effective flow cytometry. Moreover, in healthy samples, the surplus of muscle can be further subdivided for multiple downstream assays, allowing researchers to concomitantly analyze the cellular, molecular, and histological features of the same tissue. Finally, for experiments manipulating FAPs in injury models with therapeutics, well validated analyses of physical function and gait in alert and active rats are available[21–24], enabling serial assessment of muscle function without the need for termination of the animal.

A key obstacle in creating an optimized FAPs isolation protocol for the rat was antibody availability. Our initial approach sought to copy the antibody panel and gating strategy protocols widely employed in the mouse[1,7–14]. While commercially available, conjugated, flow cytometry-tested, and validated antibodies that recognize rat were available for the lineage markers CD31 and CD45, none existed for positive FAPs identifying markers Sca-1 or PDGFRα, as well as for the negative selection marker ITGA7. Unconjugated, primary antibodies specific to these markers and purported to be effective in flow cytometry were available, but of the FAPs markers Sca-1 and PDGFRα, only the Sca-1 antibody had been validated in the published literature in flow cytometric analysis of rat cells[48]. We, therefore, selected Sca-1 as the positive selection marker for FAPs. The prospect of using secondary antibodies to delineate Sca-1 and ITGA7 markers was not feasible for several reasons, but primarily because the only rat-specific antibodies for Sca-1 and ITGA7 validated for flow cytometry were generated in the same host species. Therefore, the remaining option was self-conjugation of both antibodies using commercially-available kits.

The process of choosing an optimal kit for antibody conjugation was extensive, as many kits are completely or partially intolerant to various buffer diluents (e.g., Glycine, Glycerol, BSA, Sodium Azide) present in primary antibodies, and the fluorophore provided must be compatible with the lasers equipped within the flow cytometer/cell sorter available to the investigator, and not emit at a similar wavelength to other fluorophores used to identify other cell types in the sample. The presence of diluent components in the rat-specific PDGFRα primary antibodies that were poorly compatible with the conjugation kits, was an additional consideration in the choice of Sca-1 as the positive FAPs selection marker in this protocol. While these results demonstrate that both Sca-1::APC and ITGA7::PE-Cy7 conjugations resulted in identification of distinct positive-stained populations on both compensation beads and cells, it was found that the former exhibited decay in fluorescence sooner than was advertised by the manufacturer. It is highly recommend that researchers conjugate Sca-1::APC according to the manufacturer's instructions, immediately prior to the first time use (e.g., the day before the experiment) to ensure robust signal, plan experiments accordingly such that a single batch of conjugated antibody can be used within the antibody's window of effectiveness, and validate each batch by single-staining compensation beads and titrating on single cell suspensions. More importantly, however, the critical interaction noted between Sca-1::APC and ITGA7::PE-Cy7 prevented the accurate delineation of populations of FAPs vs MPs, necessitating an alternative strategy be employed.

Boscolo Sesillo et al. recently demonstrated the successful flow cytometric isolation of rat muscle stem cells using VCAM-1 as a single positive selection marker[33] in CD31, 45 and 11b negative cells. With VCAM-1 as the MP selection marker, we utilized a novel antibody panel to identify FAPs (Lin-/Sca-1+/VCAM-1-) and MPs (Lin-/Sca-1-/VCAM-1+) and validated the approach with *in vitro* culture of FACS sorted cells from healthy gastrocnemius muscle. Freshly isolated FAPs and MPs immunostained solely for their respective alternative markers PDGFRα and Pax7. Cultured FAPs differentiated into populations of mature fibroblasts and adipocytes, and MPs into mature myocytes/myotubes. No cross contamination of FAPs and MPs within the cultures occurred. Immunostaining of histologic cross-sections confirmed infiltration of areas of fibro-fatty degradation in denervated muscle with Sca-1 positive cells.

While we undertook flow cytometric analysis of long-term denervated muscle to validate our protocol in severely atrophic, fibrotic and fat-infiltrated muscle, we incidentally observed the emergence of a FAPs sub-population with increased Sca-1 signal (denoted Sca-1 High) as compared to the baseline Sca-1 expression (denoted Sca-1 Med/Low) over time. Sca-1 High FAPs increased significantly late post-denervation (12 weeks plus), while Sca-1 Med/Low FAPs peaked early at 5 weeks before declining back to baseline levels. Heterogeneity in the FAPs phenotype has been reported[10,30]. FAPs with higher Sca-1 expression were shown to differentiate more readily into adipocytes, and when exposed to fibrogenic stimulation increased expression of Col1a1[30,49]. The dynamics of the FAPs sub-populations observed here appear to be in-line with the time-course of denervation-induced sequelae and muscle's regenerative capacity[47] and thus may characterize FAP sub-populations with differing cellular programs. Sca-1 High FAPs become up-regulated at late time-points concomitant with fibro/fatty infiltration and a decline in regenerative potential, while Sca-1 Med/Low FAPs become up-regulated during muscle's regenerative window and may aid in effective regenerative myogenesis. The FAPs isolation protocol presented here provides investigators the ability to identify and isolate these various populations for future study.

We identified a population of cells staining positive for both Sca-1 and VCAM-1 (Lin-/Sca-1+/VCAM-1+) and ascertained this double positive population to be a mixture of FAPs and MPs. Separation of this population using a third marker – ITGA7 – was not possible due to the interaction between Sca-1 and ITGA7 primary antibodies. Malecova and colleagues reported a sub-population of VCAM-1 expressing FAPs that is nearly absent in healthy muscle, transiently increased with acute inflammation, and their persistence in mdx mice (model of muscular dystrophy) was associated with chronic muscle inflammation and fibrosis[10]. In contrast, and although not a pure FAPs population, we found that the Lin-/Sca-1+/VCAM-1+ population decreased to or below baseline levels with chronic muscle fibrosis at 12- and 14-weeks post-denervation. Denervation does not induce the same inflammatory reaction and cycling regeneration attempts experienced by mdx mice, which may explain the differences in our results. Our identification of MPs in the Lin-/Sca-1+/ VCAM-1+ cell population is in keeping with the report of Sca-1 expression on a very small proportion of MPs in healthy muscle, with a transient increase following injury during myoblast proliferation and subsequent withdrawal from the cell cycle[45]. Thus, while our antibody labeling and FACS gating strategy successfully

isolates pure populations of FAPs and MPs for study, experiments requiring flow cytometry-based quantification of these populations may slightly underestimate their numbers due to the small percentage of cells in both progenitor lines that co-express Sca-1 and VCAM-1. Regardless, the current protocol clearly demonstrates the classic biphasic response of MPs to denervation injury, with short-term upregulation and subsequent depletion of the population in long-term denervated muscle. Similarly, the increase in FAPs reported in isolated shorter-term denervation injury is recapitulated here, and shown to be further sustained using the long-term tibial nerve transection model.

In summary, this protocol provides researchers with a previously unexplored animal, the rat, in which to use flow cytometric methods to simultaneously study FAPs and MPs. Experiments in mice limited by quantity of skeletal muscle, and the frequent need to undertake terminal experiments to assess muscle strength and function, can be readily conducted in the larger rat and with a more extensive range of non-lethal strength and functional assessment methods. Overall, FAPs studies in the rat may uncover novel roles of this key progenitor population in acute and chronic muscle and peripheral nerve trauma and disease, subsequently increasing the potential for developing cell-specific therapies.

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**DISCLOSURES**

The authors have no conflicts to disclose.

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Figure

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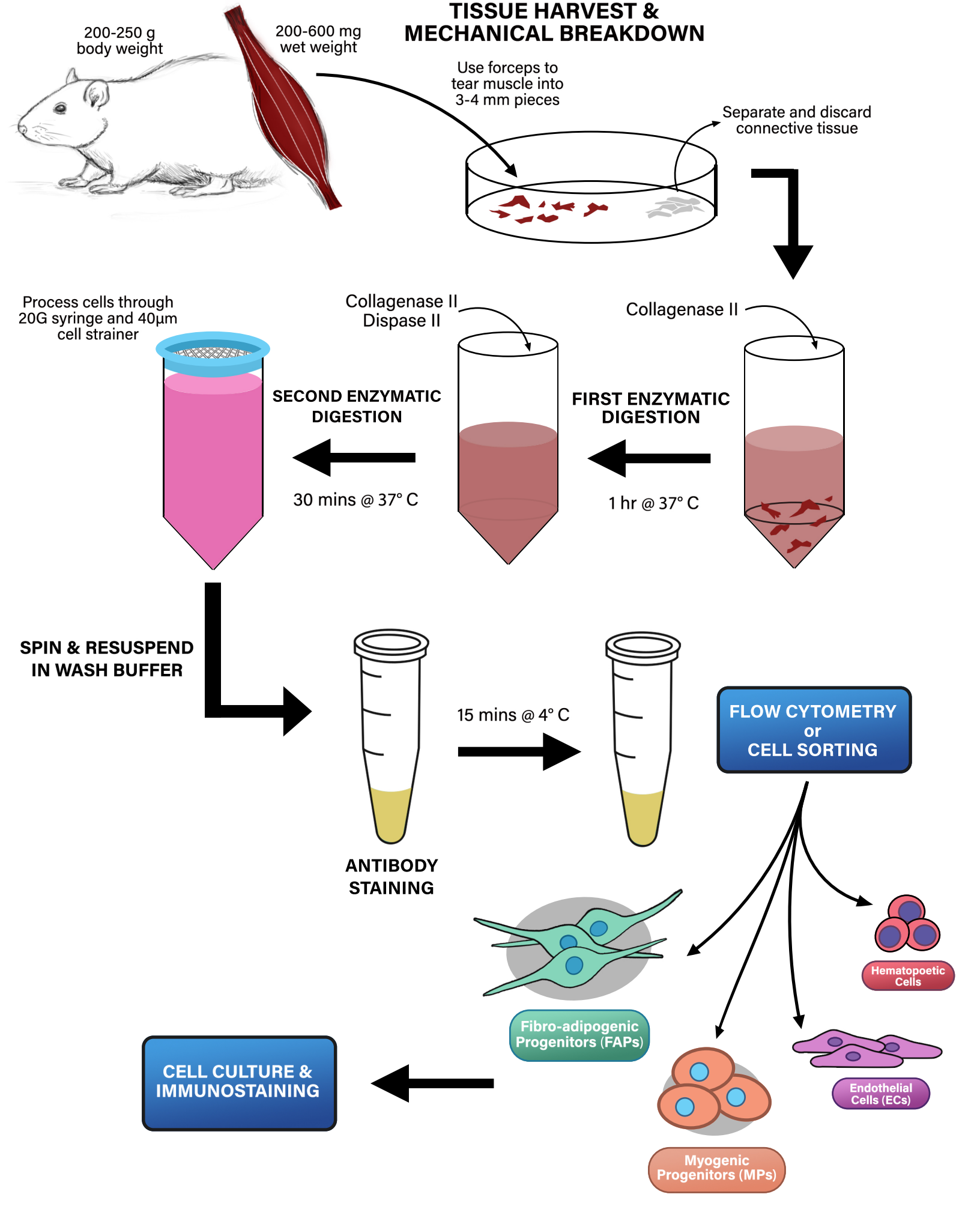

Figure

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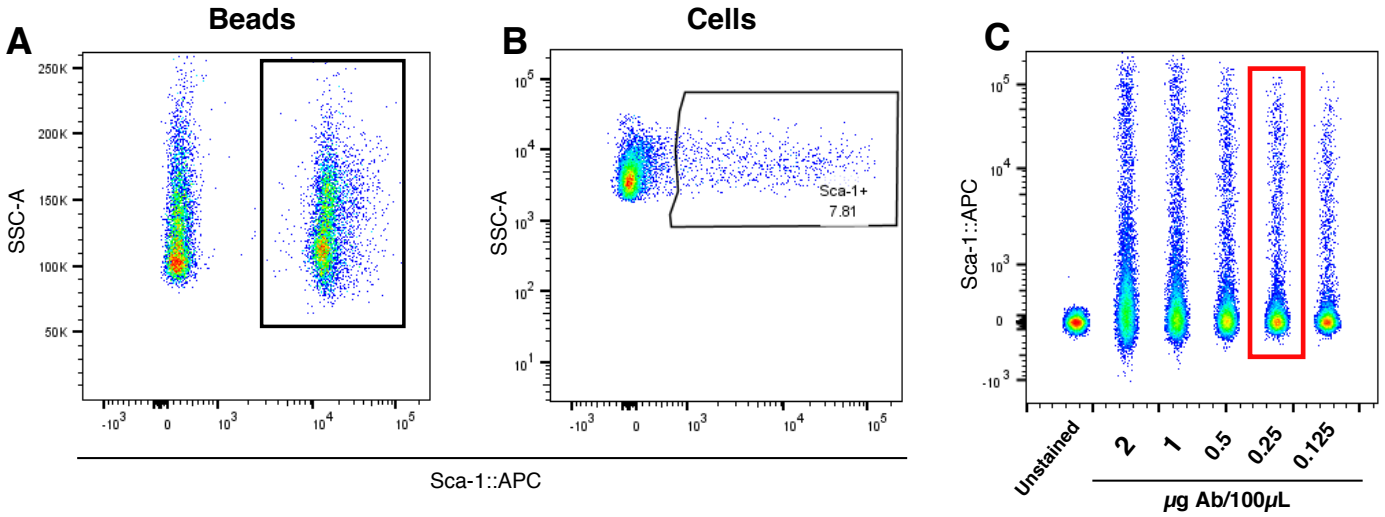
# Figure 2



Figure

# Figure 3

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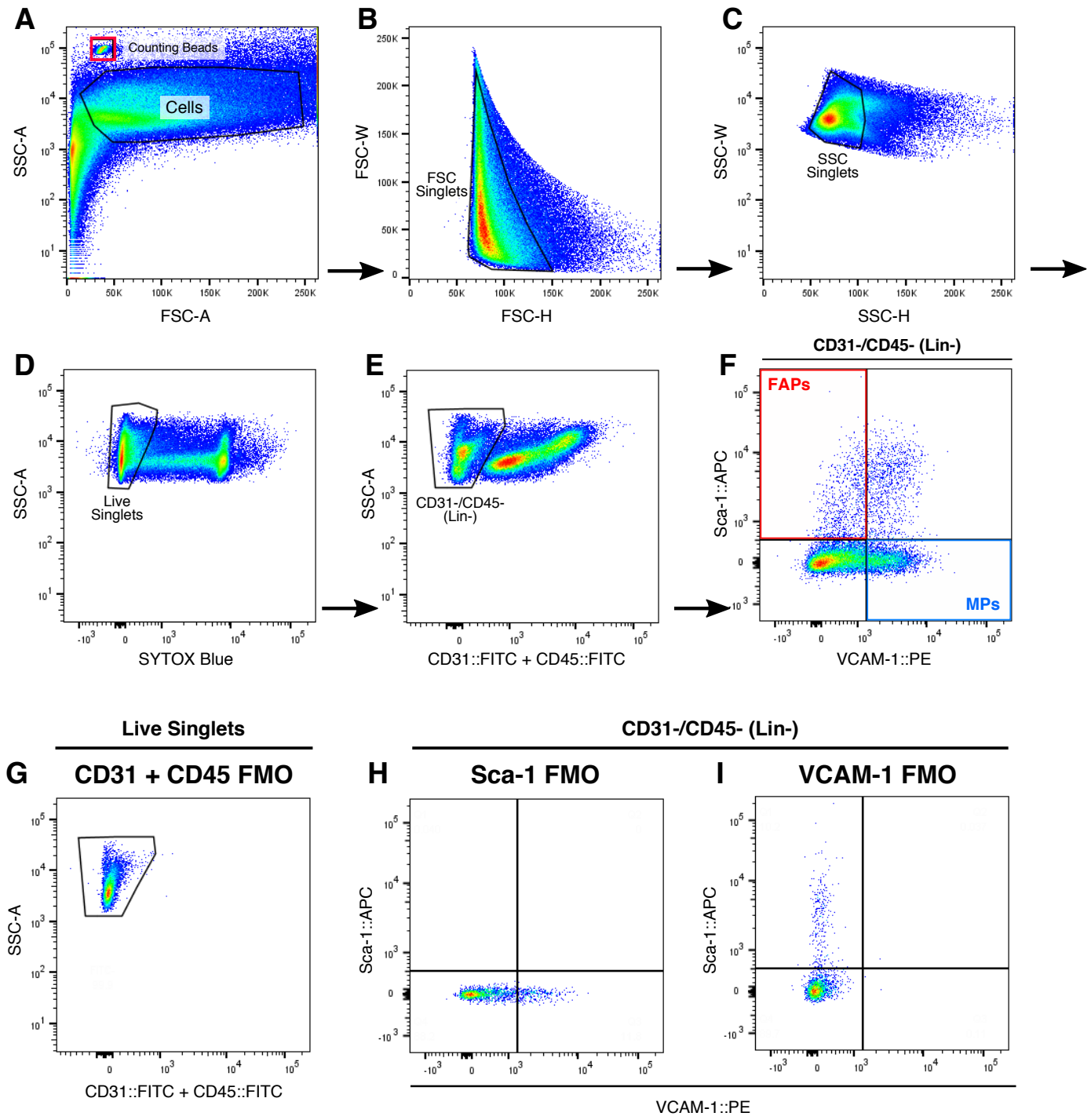
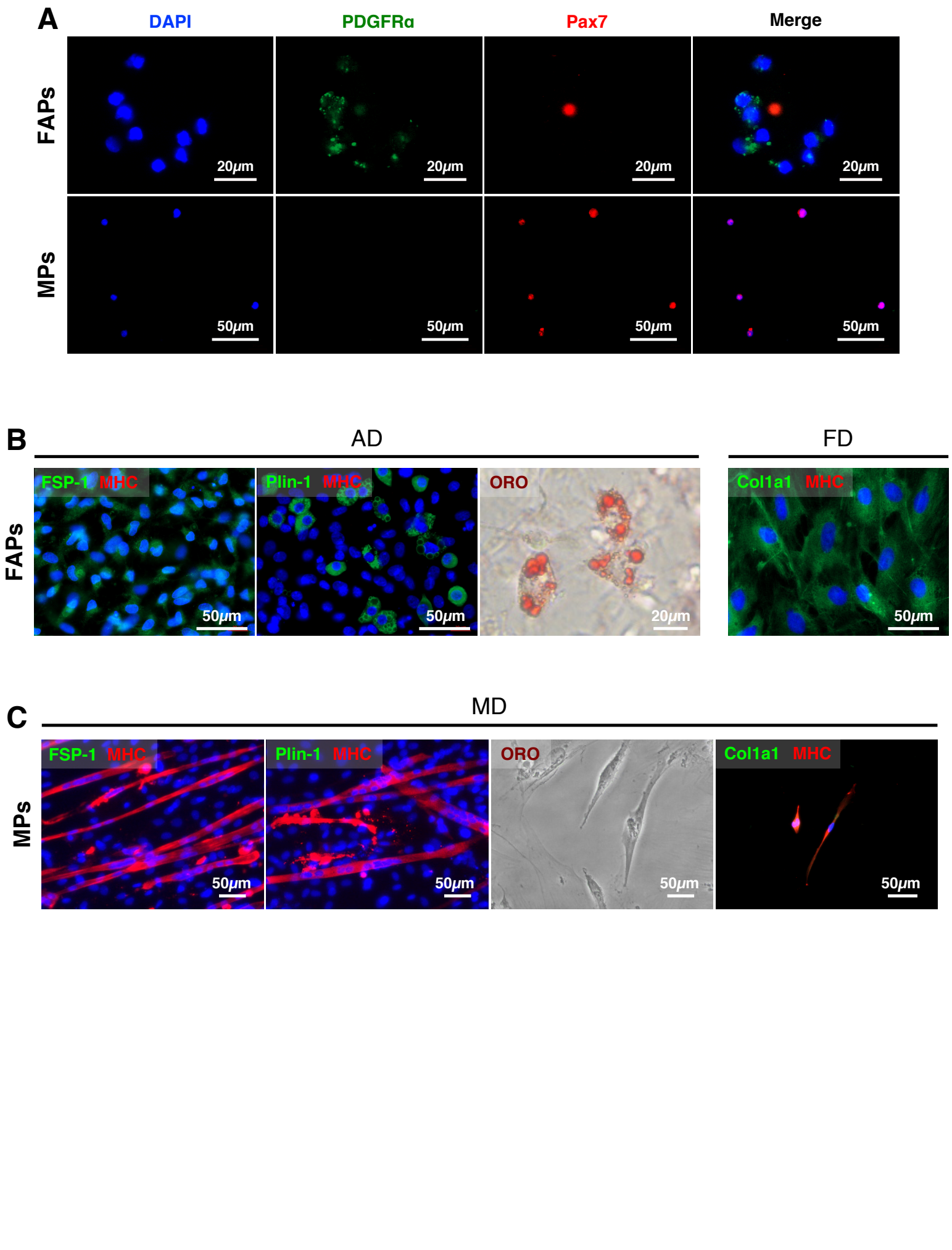


# Figure 4



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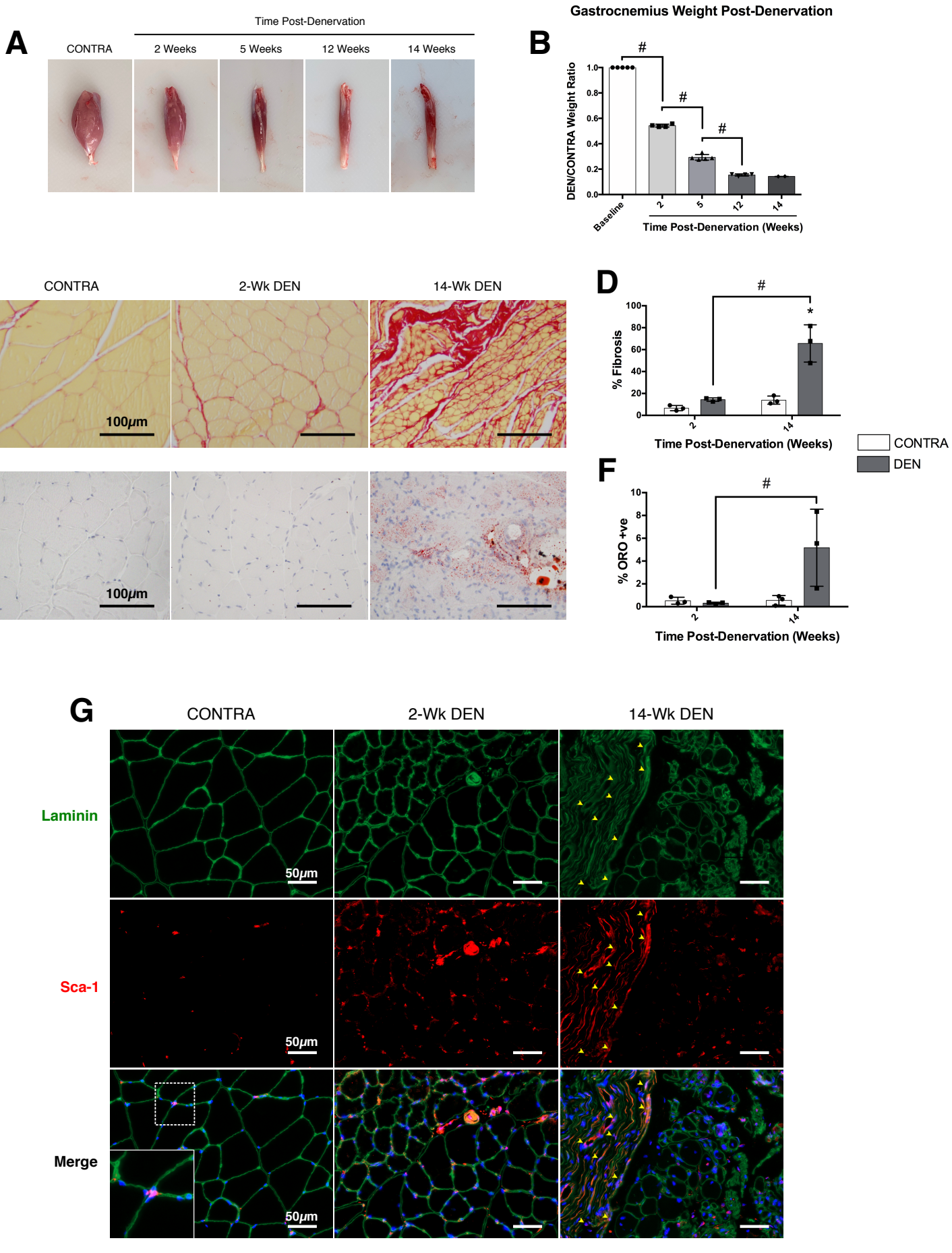

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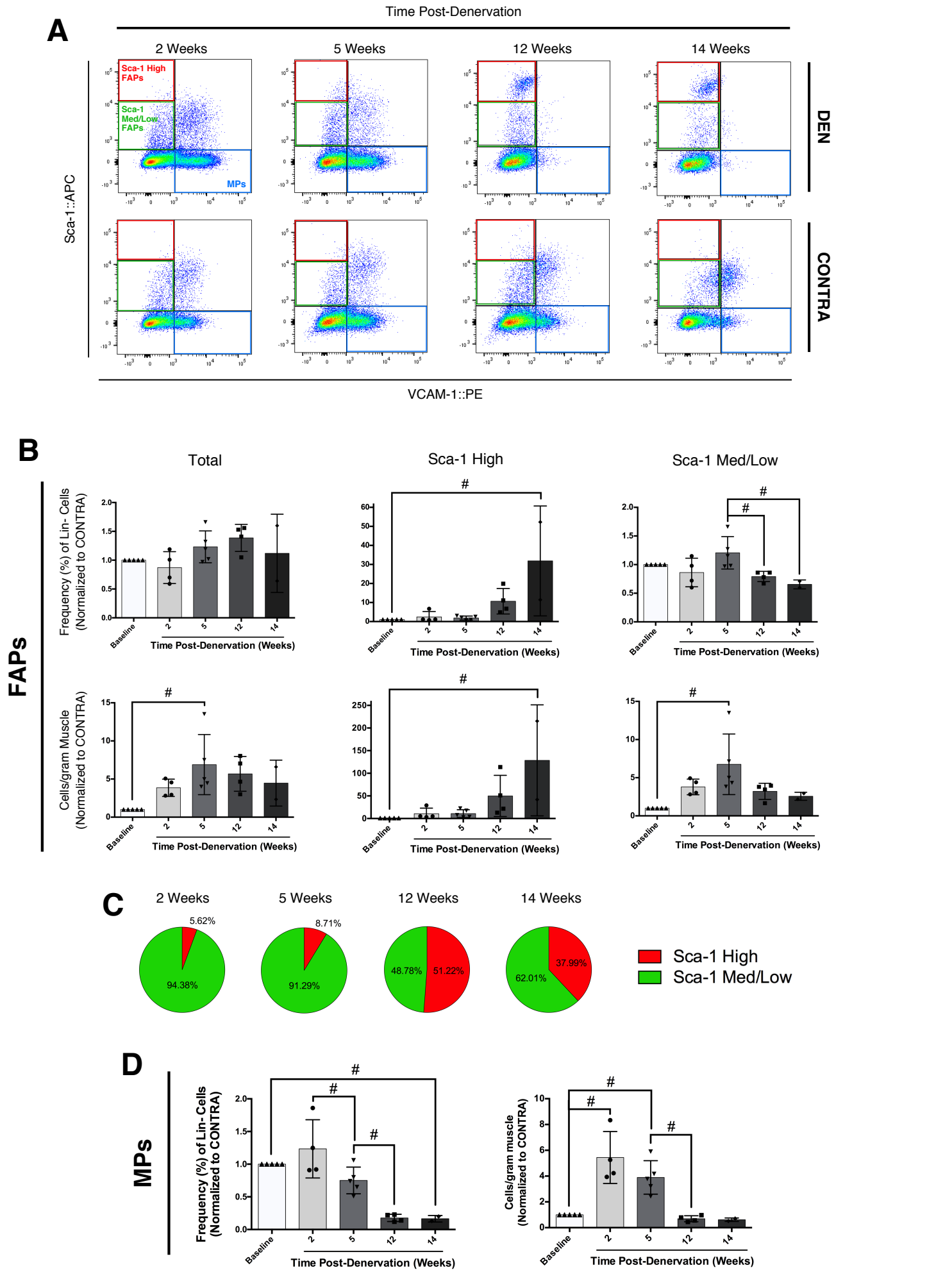

Figure

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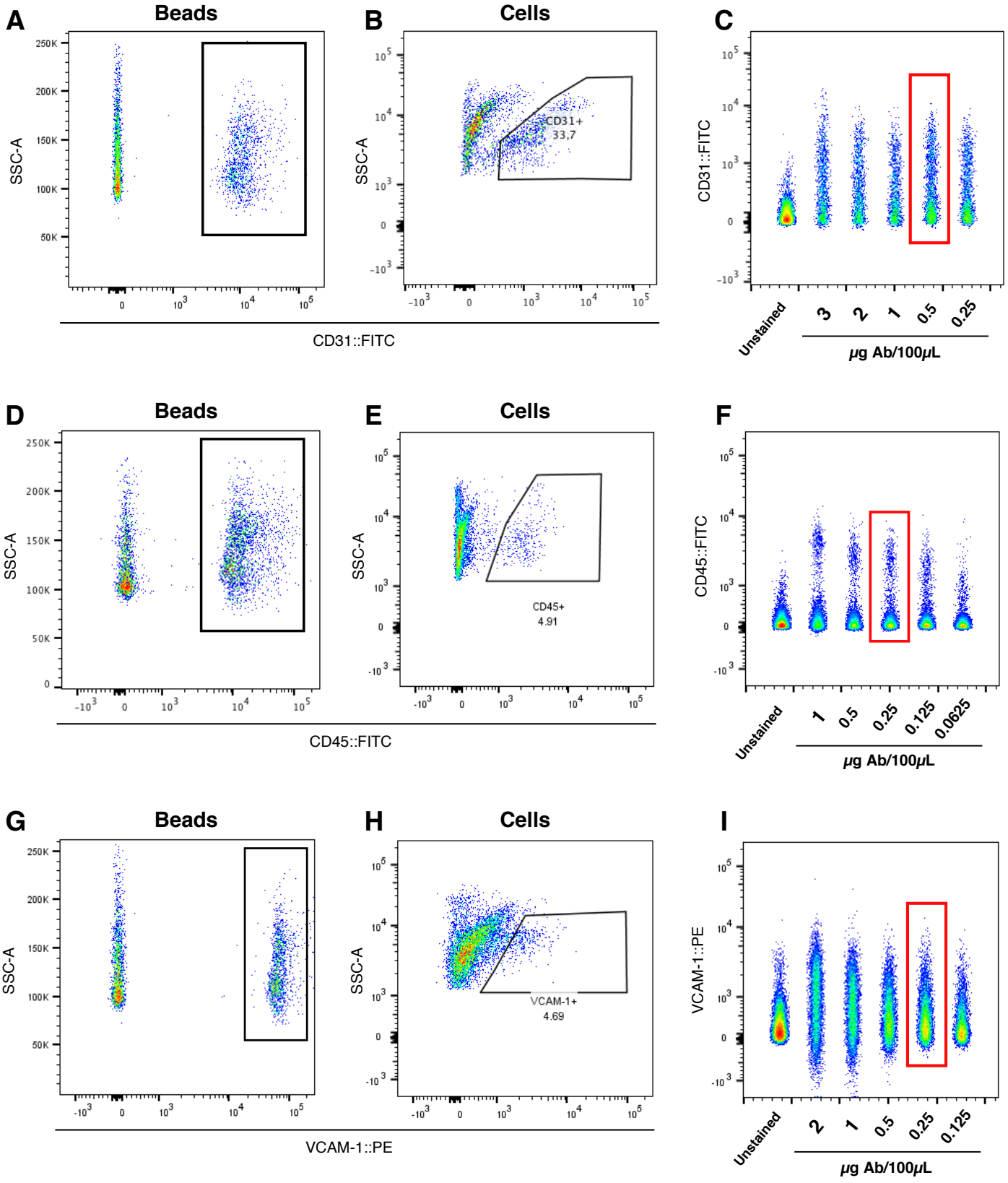
# Supplemental Figure 1



Figure

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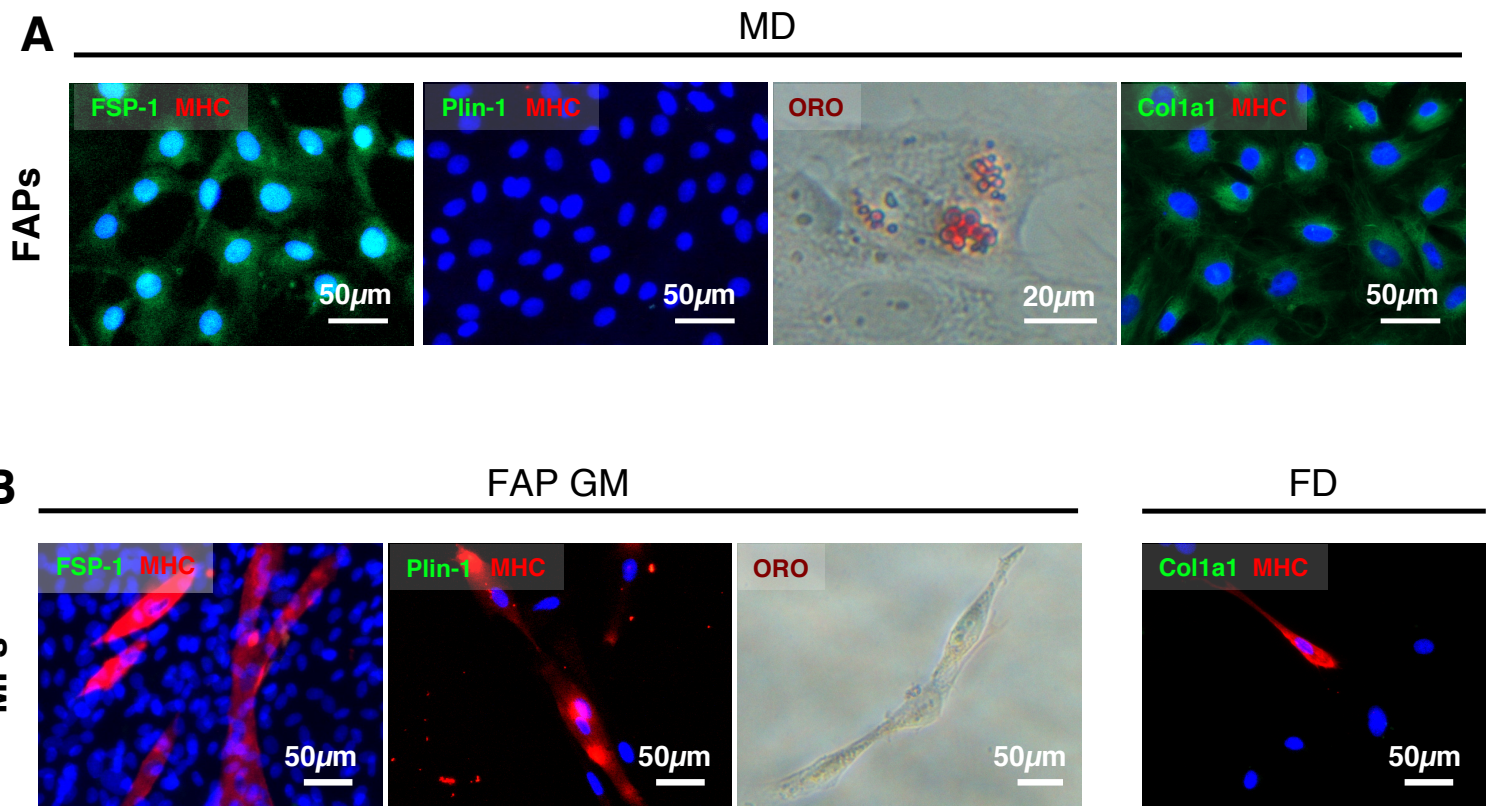
# Supplementary Figure 2



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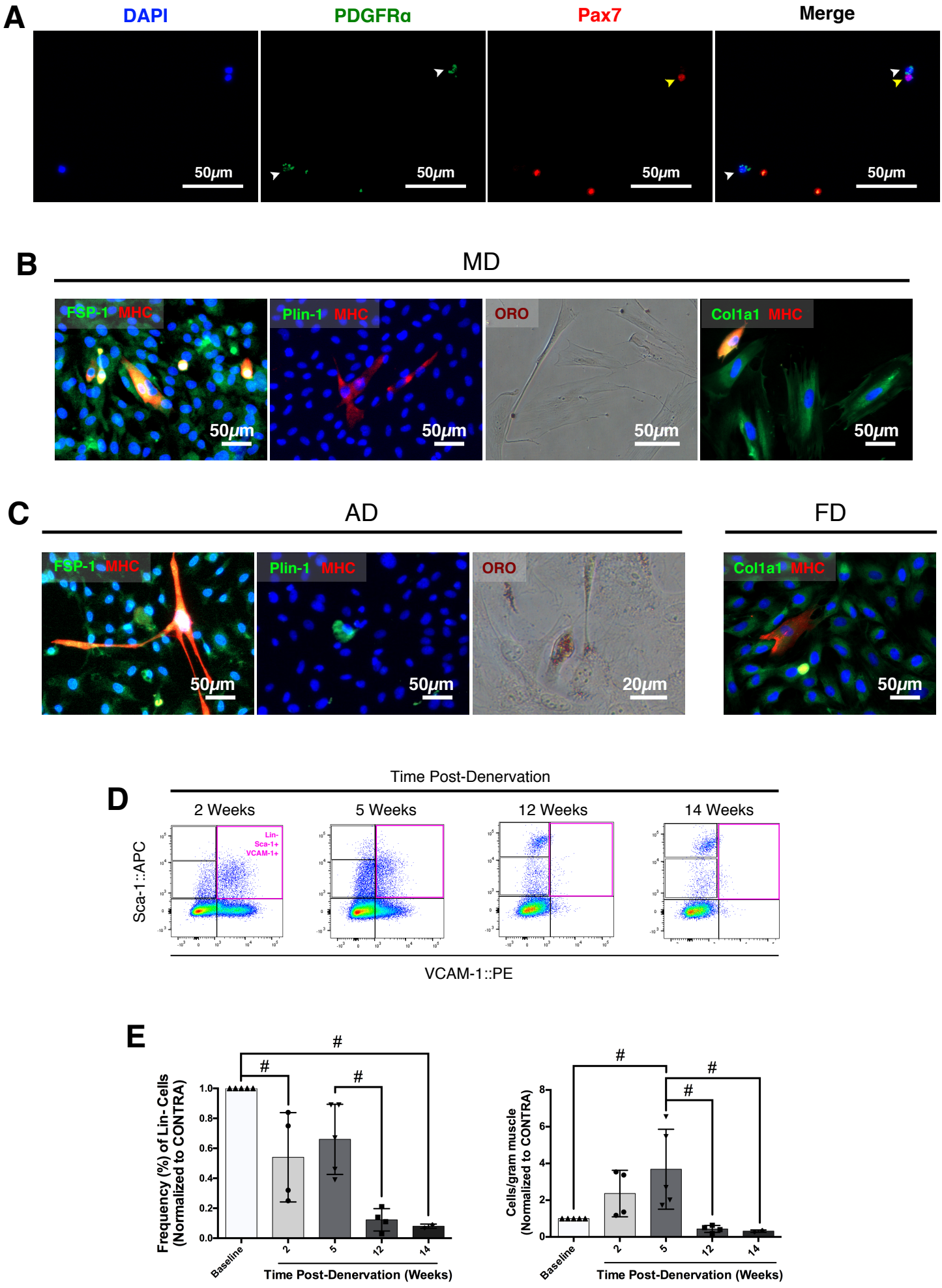

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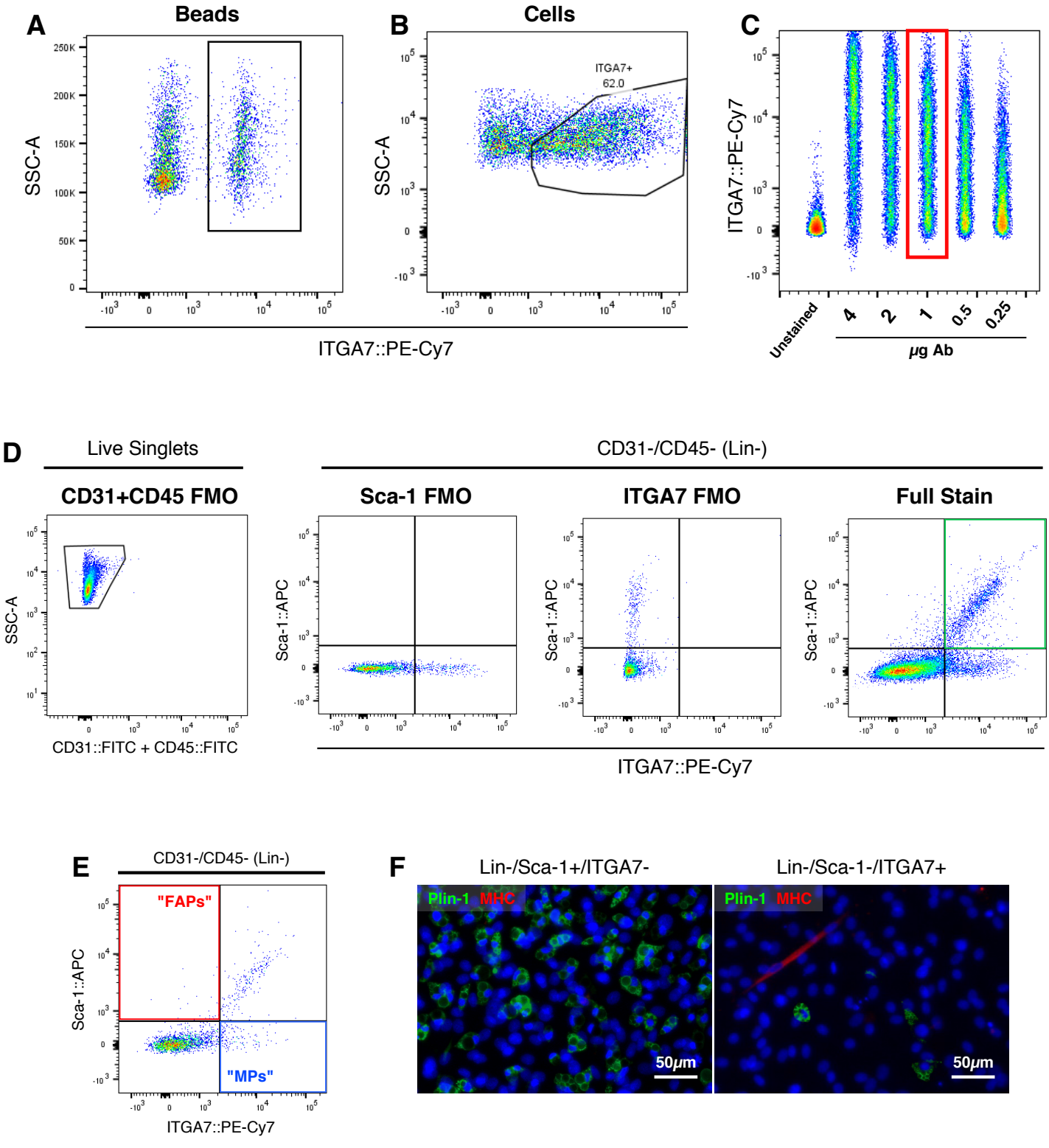
# Supplementary Figure 4



## Table 1. Flow Cytometry Antibody Staining Controls

| |
|---|
| 1. Unstained |
| 2. Viability Control |
| 3. CD31 + CD45 FMO |
| 4. Sca-1 FMO |
| 5. VCAM-1 FMO |
| 6. CD31 + CD45 Single Stain (Beads) |
| 7. CD31 + CD45 Single Stain (Cells) |
| 8. Sca-1 Single Stain (Beads) |
| 9. Sca-1 Single Stain (Cells) |
| 10. VCAM-1 Single Stain (Beads) |
| 11. VCAM-1 Single Stain (Cells) |

## Table 2. Flow Cytometry Antibody Staining Matrix

| | CD31::FITC (µg) | CD45::FITC (µg) | Sca-1::APC (µg)* | VCAM-1::PE (µg) | SYTOX Blue (µM; Final Concentration) |
|---|---|---|---|---|---|
| **Unstained Control** | | | | | |
| | -- | -- | -- | -- | -- |
| **Single-Stained Controls** | | | | | |
| SYTOX Blue (Viability Control) | -- | -- | -- | -- | 1 |
| CD31 & CD45 | 0.5 | 0.25 | -- | -- | 1 |
| Sca-1 | -- | -- | 0.25 | -- | 1 |
| VCAM-1 | -- | -- | -- | 0.25 | 1 |
| **Fluorescence Minus One (FMO)** | | | | | |
| CD31 & CD45 | -- | -- | 0.25 | 0.25 | 1 |
| Sca-1 | 0.5 | 0.25 | -- | 0.25 | 1 |
| VCAM-1 | 0.5 | 0.25 | 0.25 | -- | 1 |
| **Experimental Samples** | | | | | |
| Full Stain | 0.5 | 0.25 | 0.25 | 0.25 | 1 |

Table of Materials

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| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 5 mL Polypropylene Round-Bottom Tube | Falcon | 352063 | |
| 5 mL Polystyrene Round-Bottom Tube with Cell-Strainer Cap | Falcon | 352235 | |
| 10 cm cell culture dishes | Sarstedt | 83.3902 | |
| 12-well cell culture plate | ThermoFisher | 130185 | |
| 12 mm glass coverslips, No.2 | VWR | 89015-724 | |
| 10 mL Syringe | Beckton Dickenson | 302995 | |
| 15 mL centrifuge tubes | FroggaBio | 91014 | |
| 20 gauge needle | Beckton Dickenson | 305176 | |
| 25mL Serological pipette | Sarstedt | 86.1685.001 | |
| 40µm cell strainer | Fisher Scientific | 22363547 | |
| 50mL centrifuge tubes | FroggaBio | TB50 | |
| AbC Total Antibody Compensation Beads | ThermoFisher | A10497 | |
| Ammonium Chloride, Reagent Grade | Bioshop | AMC303.500 | |
| APC Conjugation Kit, 50-100µg | Biotium | 92307 | |
| Aquatex Aqueous Mounting Medium | Merck | 108562 | |
| Biolaminin 411 LN | Biolamina | LN411 | |
| Bovine Serum Albumin (BSA) | Bioshop | ALB001 | |
| Calcium Chloride | Bioshop | CCL444.500 | |
| Collagenase Type II | Gibco | 17101015 | |
| CountBright Plus Absolute Counting Beads | ThermoFisher | C36995 | |
| Dexamethasone | Millipore Sigma | D4902 | |
| Dispase | Gibco | 17105041 | |
| Dulbecco's Modified Eagle Medium (DMEM) (1X) | Gibco | 11995-065 | (+)4.5 g/L D-Glucose<br>(+)L-Glutamine<br>(+)110 mg/L Sodium Pyruvate |
| EDTA | FisherScientific | S311 | |
| FACSClean Solution | Beckton Dickenson | 340345 | |
| FACSDiva Software | Beckton Dickenson | -- | |
| FACSRinse Solution | Beckton Dickenson | 340346 | |
| Fetal Bovine Serum | Sigma | F1051 | |
| Flow Cytometry Sheath Fluid | Beckton Dickenson | 342003 | |
| FlowJo Software | Beckton Dickenson | -- | |
| Fluorescent Mounting Medium | Dako | S302380-2 | |
| Goat anti-mouse Alexa Fluor 555 secondary antibody | Invitrogen | A21424 | |
| Goat anti-rabbit Alexa Fluor 488 secondary antibody | Invitrogen | A11008 | |
| Goat anti-rabbit Alexa Fluor 555 secondary antibody | Invitrogen | A21429 | |
| Goat Serum | Gibco | 16210-064 | |
| Ham's F10 Media | ThermoFisher | 11550043 | (+) Phenol Red<br>(+) L-Glutamine<br>(-) HEPES |
| Hank's Balanced Salt Solution (HBSS) (1X) | Multicell | 311-513-CL | |
| Heat Inactivated Horse Serum | Gibco | 26050-088 | |
| Hemocytometer | Reichert | N/A | |
| HEPES, minimum 99.5% titration | Sigma | H3375 | |
| Horse Serum | ThermoFisher | 16050130 | |
| Human Transforming Growth Factor β1 (hTGF-β1) | Cell Signaling | 8915LF | |
| Humulin R | Lilly | HI0210 | |
| IBMX | Millipore Sigma | I5879 | Also known as 3-Isobutyl-1-methylxanthine |
| Isopropanol | Sigma | I9516 | Also known as 2-propanol |
| Lewis Rat, Female | Charles River Kingston | 004 (Strain Code) | 200-250 grams used |
| LSRFortessa X-20 Benchtop Cytometer | Beckton Dickenson | -- | |
| Microcentrifuge | Eppendorf | EP-5417R | |
| MoFlo XDP Cell Sorter | Beckman Coulter | -- | |
| Mouse Anti-CD31::FITC Antibody | Abcam | ab33858 | Clone TLD-3A12 |
| Mouse Anti-CD45::FITC Antibody | Biolegend | 202205 | Clone OX-1 |
| Mouse Anti-CD106::PE Antibody | Biolegend | 200403 | Also known as VCAM-1 |
| Mouse Anti-MHC Antibody | Developmental Studies Hybridoma Bank (DSHB) | N/A | Also known as MF20 |
| Mouse Anti-Pax7 Antibody | Developmental Studies Hybridoma Bank (DSHB) | N/A | |
| Neutral Buffered Formalin, 10 % | Sigma | HT501128 | |
| Oil Red O | Millipore Sigma | O0625 | |
| PE-Cy7 Conjugation Kit | Abcam | ab102903 | |
| Penicillin-Streptomycin | Sigma | P4333 | |
| Phosphate Buffered Saline, pH 7.4 (1X) | Gibco | 10010-023 | (-)Calcium Chloride<br>(-)Magnesium Chloride |
| Potassium Bicarbonate, Reagent Grade | Bioshop | PBC401.250 | |
| Rabbit Anti-Fibroblast Specific Protein 1 (FSP-1) Antibody | Invitrogen | MA5-32347 | FSP-1 also known as S100A4 |
| Rabbit Anti-Integrin-a7 Antibody | Abcam | ab203254 | |
| Rabbit Anti-Laminin Antibody | Sigma | L9393 | |
| Rabbit Anti-Perilipin-1 Antibody | Abcam | ab3526 | |
| Rabbit Anti-Sca-1 Antibody | Millipore Sigma | AB4336 | |
| Rabbit Recombinant Anti-Collagen Type I Antibody | Abcam | ab260043 | Also known as Col1a1 |
| Rabbit Recombinant Anti-PDGFR Alpha Antibody | Abcam | ab203491 | |
| Recombinant Human FGF-basic | Gibco | PHG0266 | |
| Sodium Azide | Sigma | S2002 | |
| Triton-X-100 | Fisher Scientific | BP151 | |
| Troglitazone | Millipore Sigma | T2573 | |
| Tween-20 | Bioshop | TWN510 | |

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March 11 2021

Dear Dr Bajaj

Please find the revision of our manuscript entitled " Identification, isolation, and characterization of Fibro-adipogenic progenitors (FAPs) and Myogenic Progenitors (MPs) in skeletal muscle in the rat ," submitted for consideration of publication in the Journal of Visualized Experiments.

We would first like to thank the reviewers for a thorough and very helpful review of the manuscript. We have completed new studies to validate the purity of the isolated populations of FAPs and MPs including further immunocytochemistry experiments for alternative markers (PDGFRα, Pax7) of these cells, in addition to culturing them in fibrogenic, adipogenic and myogenic media. We have also characterized our Lin-/Sca-1+/VCAM-1+ population, and re-analyzed our data, quantifying the frequency of the populations, in addition to reporting absolute cells counts/gm of muscle. Finally we have revised the introduction and discussion to i) address the broader nomenclature of the FAPs population as mesenchymal or stromal progenitors, ii) the use of PDGFRα as a common marker of the FAPs population and iii) to clarify the novelty of evaluating FAPs in *isolated* long-term denervated injury in the rat. Below we have provided a point-by-point response to the editorial and reviewers' comments.

We believe the revisions made now provide a robust protocol for FAPs identification and isolation and substantially improve the manuscript. We feel this will be of great interest to researchers studying muscle regeneration and function following injury or in disease. We hope it is now suitable for publication.

Sincerely,

Jane Batt MD PhD

**Editorial comments:**

*(C1). Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues*.
(R1). Manuscript has been checked for spelling and grammar

*(C2). Please remove the alphabets preceding the headings of the manuscript.*
(R2). Letters are removed.

*(C3). JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., FACSDiva, FlowJo, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.*
(R3). All commercial language has been removed from manuscript body and placed in Table of Materials.

*(C4). Please use standard abbreviations for time throughout the protocol (e.g., 5 min, 4 h).*
(R4). All abbreviations have been standardized.

*(C5). Line 190/201: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).*
(R5). Centrifuge speeds have been converted from rpm to x g.

*(C6). Line 206: Please use capital G with single spacing when mentioning needle/catheter gauges. Examples: 21 G, 30 G needle*
(R6). Needle gauge has been appropriately notated to G.

*(C7). Line 320-455: Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.*
(R7). Numbering has been adapted to follow the JoVE Instructions for Authors.

*(C8). Line 326-401: Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc.) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.*

(R8). Steps 5.2-5.3 in the protocol have been supplemented with more specific user actions to delineate what cell populations are being identified and sorted at each gating stage. Please note however that the operation of the flow cytometer or cell sorter will be dependent on the equipment manufacturer, model and the operational software (program and version) – so the request for directions in the protocol regarding "button clicks for software action" will not be universally applicable. We have instead ensured that the gating strategy is now clearly and adequately detailed in the protocol so that users trained on any flow cytometer or cell sorter (makes or models) with any operational software can do so. We have also stated that individuals who do not know how to use flow cytometers and cell sorters, receive training or work with an individual familiar with the operation of the equipment to conduct the gating as we have described.

*(C9). The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.*
(R9). Protocol has been revised to include only action items.

*(C10). Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.*
(R10). Three pages of the protocol have been highlighted.

*(C11). Please move the Figure Legends section to the end of the Representative Results.*
(R11). Figure legends section has been moved to end of Representative Results.

*(C12). Please rename the Material Supplemental as Supplementary file and refer it in the protocol.*
(R12). All instances of "Materials Supplemental" have now been referred to as "Supplementary File" in the manuscript text.

*(C13). Please do not use the &-sign or the word "and" when listing authors. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. End the list of authors with a period. Example: Bedford, C. D., Harris, R. N., Howd, R. A., Goff, D. A., Koolpe, G. A. Quaternary salts of 2-[(hydroxyimino)methyl]limidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998). Please do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations*
(R13). Reference formatting has been changed.

*(C14). Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.*

(R14). All trademark and registered symbols have been removed from the Table of Materials.

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**Reviewers' comments:**

**Reviewer #1:**

*In this manuscript Te and colleagues describe a method to isolate Fibro Adipogenic Progenitors (FAPs) from rat skeletal muscle upon denervation. Cytofluorimetric characterization of Rat muscle resident cell population is poorly described in literature; for this reason, the manuscript might be a valuable resource for the muscle research community.*

*(C1) In the revised version of the manuscript the authors use Vcam1 antibody (replacing the less efficient a7intgrin Ab) to stain myogenic cells. I really appreciate the effort of the authors to reproduce all the experiments with the new antibody as well as the inclusion of new analyses (i.e. Red Oil Staining). The authors fully accomplish the referee's queries and the revised version of the manuscript has been improved and it is now ready to published on JoVE.*

(R1) Thank-you. We appreciative the reviewer's critique, which helped us to optimize the protocol and improve the manuscript substantially.

**Reviewer #2:**

*Manuscript Summary:*

*(C1) The revised manuscript now suitable for publication*

(R1) Thank-you. We appreciate the review and helpful comments for revision.

**Reviewer #3:**

*Manuscript Summary*

*Overall comments*

*The authors describe a method for isolation of non- myogenic progenitors (termed herein FAPs) and myogenic progenitors from non-injured and denervated rat skeletal muscle. In house conjugated anti-rat sca-1 is successfully used to identify sca-1+ cells non-injured and injured rat muscle. Sca-1 and VCAM-1 are used to identify and sort two muscle residing cell populations and changes in their frequency post injury by flow cytometry.*

*Overall, this study adds to what is known so far about skeletal muscle denervation in human and similar experimental models in rodents and supports the notion that muscle denervation is linked to progressive fibrotic degeneration. Applying sca-1 and VCAM-1 cell sorting to rat muscle will be of interest to other scientists.*

*(C1). However, the conception of the novelty of the described model and FAP characterization is "enlarged". The novelty of rat denervation model is somewhat diminished by the fact that multiple previous publications demonstrate the effects of long-term denervation on skeletal*

*muscle (e.g. reviewed by Carlson Eur J Transl Myol. 2014, Lee et al, Sci Rep, 2018). Additionally, the effect of long-term denervation on shoulder muscle FAPs was also previously described in mouse (Jensen et al JSES 2018, Mosich et al JCI Insight 2019) and rat (Lee et al, Sci Rep, 2018).*

(R1). We thank the reviewer for their careful evaluation and suggestions that have helped us to improve the manuscript substantially.

We agree with the reviewer that many studies have evaluated effects of long-term denervation injury on muscle, such as the Carlson publication cited above (and had acknowledged this in our original submitted manuscript) but FAPs dynamics is not addressed in this publication. We respectfully note that the additional cited references (Lee et al, 2018; Jensen et al, 2018; Mosich et al, 2019) importantly utilize a "rotator cuff tear" model which involves tendon transection of the infraspinatus and supraspinatus muscles with or without concurrent transection of the suprascapular nerve. Tendon transection and denervation are different injuries resulting in different physiologic and molecular responses. While denervation results in loss of contraction in all muscle distal to the injury, there is no direct damage to the muscle and it can still experience loading and passive stretch as the animal moves. In contrast tendon transection is a direct insult and removes all passive stretch and loading. Studies (e.g. Joshi et al J Orthop Res. 2014 January; 32(1)) report the engagement of different cellular signalling in the muscle dependent upon whether the injury is tendon disruption or denervation. These different upstream stimuli may impact the FAPs response (e.g. temporality of recruitment, phenotype) differentially. In addition, the FAPs evaluation in rat by Lee et al was limited to immunohistochemistry. They did not undertake flow cytometric identification or FACS separation of FAPs, and therefore were unable to characterize the cells in detail with respect to cellular/molecular analyses. Therefore, we believe delineating the FAPs time-course via flow cytometry/FACS in the rat following the sole insult of chronic traumatic denervation is not well characterized and remains novel. We have however, revised the manuscript to clarify our statements and specifically address the fact that FAPs recruitment has been evaluated in combination injury models besides solitary traumatic denervation injury (revised manuscript introduction paragraph 5). We also explain that it is important to study solitary chronic traumatic denervation since this is an injury in heavy industry, agriculture and even newborns (neonatal brachial plexus palsy), with significant morbidity.

*(C2). The major issue that limits the overall impact of the study in the field is the use of sca-1 and not PDGFR$\alpha$ (CD140a) which is broadly used for FAP identification in human, mouse and rat. The authors should at least relate to this point in the discussion.*

(R2). We have amended the manuscript (introduction paragraph 3 and discussion paragraphs 2 and 3) to discuss the use of PDGFR$\alpha$ as a positive FAPs selection marker and to explain our choice of Sca-1 instead of PDGFR$\alpha$ in detail. Although PDGFR$\alpha$ has been used to identify FAPs in the rat (Lee et al, 2018), as noted above it was done so not by flow cytometric analysis, but by chromogenic immunohistochemistry on tissue histologic cross-

sections. When searching for flow cytometry validated PDGFRα antibodies specified to recognize rat, the only ones available were unconjugated and suboptimal for self-conjugation due to presence of incompatible buffer constituents or poorly tolerated constituents that increase the difficulty of, and diminish the chance for, successful conjugation. In contrast, the lone available Sca-1 antibody had been validated and used successfully in flow cytometric analysis of rat cells (Kennedy et al, 2014). Due to these reasons, we chose to use Sca-1 instead of PDGFRα as a positive selection marker for FAPs.

*(C3). The term FAPs should be reconsidered: lessons from studies in human and mouse or tissues other than muscle demonstrate that sca-1 demarcates cell subset that is also capable of osteogenic differentiation. As such, and unless demonstrated otherwise by the authors, the sorted sca-1+ subset should be named stromal progenitors.*
(R3). We agree with the reviewer that the population of mesenchymal progenitor cells that lie in the stromal space between myofibers and originally designated as FAPs have been show to be capable of alternative lineage differentiation (i.e. osteogenic). This fact is highlighted in several recent review articles (e.g. M. Wosczyna et al Dev Cell 2018, B. Biferali et al Front Physiol 2019, A. Uezumi et al Front Physiol 2014) and research manuscripts (e.g. B. Malecova Nat Commun 2019). Either Sca-1 or PDGFRα are commonly employed as the positive selection marker for these cells after the exclusion of blood and endothelial cells in multiple published studies in mice, and in these manuscripts they continue to be referred to as "FAPs", as opposed to "mesenchymal" or "stromal" progenitors (e.g. Guiliani et al Cell Death Disease 2021, Madaro et al Nat Cell Biol 2018). We do agree with the reviewer that mesenchymal or stromal progenitors is likely a better designation, but FAPs currently remain a popular and widely used nomenclature in the literature. To ensure that our protocol is seen by those in the research community interested in these progenitors, we have continued to refer to them by the "FAPs" designation. However, we now make it clear in the introduction that FAPs are indeed mesenchymal progenitors with the capacity to differentiate to lineages besides adipocytes and fibroblasts, have added this term to the "Key word" list, and return to it in the discussion.

(*C4). Moreover, there is no demonstration of adipogenesis in injured muscle at any time point post denervation to support the necessity of the experiments related to adipogenic differentiation*.
(R4). Long term denervated muscle is recognized to undergo fibro-fatty degradation. We have now performed Oil Red O (ORO) staining on contralateral healthy and denervated rat gastrocnemius cross-sections harvested 2- or 14-weeks post-denervation, to confirm the fatty infiltration of long-term denervated muscle (new Figure 5E).

*(C5). Lineage differentiation set of studies should be completed and essentially the missing experiments of induced fibrogenic differentiation of sca-1+ cell subset should be added*.

(R5). We have performed induced fibrogenic differentiation of the sorted cell subsets and immunostained for Collagen type 1 (new Figure 4B and new Supplementary Figures 2B and 3C). We demonstrate collagen staining in sca-1+ cells. There are contradictory reports in the literature as to what extent myogenic progenitors (MPs)/myoblasts produce collagen 1a1 (e.g. Uezumi et al J Cell Sci 2011; Chapman et al Am J Physiol Cell Physio, 2017.). We did not see collagen expression in our MP cultures. We reference and briefly discuss this contradictory literature in our results section ( "*Validation of identification of FAPs and MPs by FACS and cell culture*" paragraph 4).

*(C6). Finally, immunolabeling for sca-1 in healthy and injured muscle should be performed as a complementary experiment that will validate sca-1 cells localization in fibrotic scars*.
(R6). We have performed Sca-1 immunohistochemistry on contralateral healthy and denervated rat gastrocnemius cross-sections harvested at 2- or 14-weeks post-denervation, and demonstrate the presence of Sca-1+ cells localized to areas of fibrosis in 14-week denervated muscle (new Figure 5G).

Title
*(C7). The manuscript describes identification, isolation and characterization of two muscle cell subsets: FAPs and MP. The title can be revised to better identify the study. The meaning of "dynamics" is a bit obscure in this context.*
(R7). Thank-you. We have modified the title to better reflect the study, as suggested

Introduction
*(C8) The sentence "FAPs have the potential to differentiate into fibroblasts, adipocytes, osteoblasts and chondrocytes" is somewhat confusing. FAPs should be defined as a subset of multipotent stromal cells that is capable of differentiation into adipogenic and fibrogenic lineages. Otherwise these are either less committed progenitors or alternatively the markers that are used for progenitor cell isolation typifies a mixed populations of progenitor cells that can give rise to fibroblasts, adipocytes, osteoblasts and chondrocytes. This should be tested.*
(R8) As noted in comment 3 (C3) above, further study of the cell population originally isolated and identified as "FAPs" by other investigators recognized their ability to differentiate beyond the fibrogenic and adipogenic lineages. Better nomenclature for these cells would be mesenchymal or stromal progenitors. However, they remain commonly referred to throughout the literature as FAPs. We therefore now discuss this in the manuscript introduction, include "mesenchymal progenitors" in the key words, but continue to use the FAPs designation to ensure our protocol is seen by researchers in this field.

*(C9). It would add to mention that in mouse muscle, either non-injured or denervated, PDGFR$\alpha$ is used to demarcate non-myogenic FAPs. Relevant citations should be Jensen et al, JSES, 2018 PMID: 29653843 and Mosich et al, JCI Insight, 2019 PMID: 31852842. It seems*

*relevant to introduce PDGFR$\alpha$ as FAP marker in human, mouse and rat muscle (Lee et al, Sci Rep, 2018 PMID: 32157195).*
(R9). We agree. We have revised the manuscript (introduction paragraph 3 and discussion paragraphs 2 and 3) to discuss PDGFR$\alpha$ as a positive selection marker of FAPs, and include these references.

*(C10). Major - The authors state that: "Studies delineating the long-term dynamics of FAPs are currently limited to Duchenne's muscular dystrophy, using the well-studied mdx mouse model3-5. FAPs in the context of injury models such as muscle denervation have not been well characterized; only a few studies to date have investigated traumatic denervation, and these have been consistently limited to a short-term time frame5,21". This statement should be corrected. There are multiple studies demonstrating the direct long-term (up to 10 weeks) contribution of FAPs to development of fibrosis and fat accumulation in the shoulder muscle (rotator cuff) following denervation: Jensen et al, JSES, 2018 PMID: 29653843 and Mosich et al, JCI Insight, 2019 PMID: 31852842, Lee et al, Sci Rep, 2018 PMID: 32157195 and additional publications by Feeley et al, Mendias et al and several other research groups.*
(R10). Please see our response to comment 1 (C1) above. We apologize for not clarifying that we were considering isolated denervation injury. The cited studies evaluate FAPs in the massive rotator cuff model, where concomitant tendon rupture and nerve transection are performed and studies looking at isolated nerve transection in mice are carried to 1 month. We have corrected this statement to clearly identify the fact that we are referring to FAPs analysis as the sole insult of long-term traumatic denervation. In the event we have missed publications in our literature search we have now tempered our statement to read "The response of FAPs to the sole insult of chronic traumatic denervation, a common occurrence in work-place accidents in heavy industry, agriculture, and in birth traumas (brachial plexus injury) with significant morbidity, has not been as well characterized, often limited to a short-term time frame" (revised manuscript introduction, paragraph 5).

Protocol
*(C11). Major - Calculations of subset frequencies out of parent population of CD31-CD45- should be added*
(R11). We have calculated the subset frequencies of each population out of CD31-/CD45- cells and have included them in a new Figure 6 and Supplementary Figure 3. Although it is common to report the flow cytometry results as frequencies, the use of counting beads additionally provides an accurate delineation of population counts when utilizing a non-volumetric flow cytometer, as was used in this protocol (Saraiva et al, Cytometry Part B, Clinical Cytometry 2019; Villarroya-Beltri et al, Method is Molecular Biology 2013). Hence we also present our original quantification of the cells per gram muscle.

*(C12). Major - Section 5B.4 - induced adipogenic differentiation should be also applied to MP and induced myogenic differentiation should be applied to "FAPs" for evaluation of the*

*phenotypic uniformity of the sorted populations. This is an essential experiment for marker validation.*
(R12). We have performed these additional experiments, in which both FAPs and MPs are subjected to "alternative" media. FAPs are now subjected to induced myogenic differentiation, and MPs are subjected to each of fibrogenic and adipogenic induced differentiation. We found no evidence of FAPs contaminating the MPs nor MPs contaminating the FAPs (new Supplementary Figure 2).

*(C13). Induced fibrogenic differentiation experiments are missing, and collagen production should be measured as well in fibrogenic cultures.*
(R13). Please see comment 5 (C5) above. We have completed induced fibrogenic differentiation experiments and immunostained for collagen 1, rather than measuring collagen production (new Figure 4 and Supplementary Figure 2)

Results and Figures
*(C14). Major - What is the frequency of sca-1/VCAM-1 subsets within gated CD31-CD45- live cells in all tested time points in healthy and injured muscle? Analysis of the frequency of sca-1-high and sca-1-Med/low subsets is also relevant and can be easily conducted and discussed (see Pannerec et al, Development, 2013).*
(R14). Thank-you for this suggestion to separate out the sca-1 High and sca-1 Med/Low subsets. We now report the frequencies, as well as the number of cells per gram of muscle, for the Sca-1 total, Sca-1 High and Sca-1 Med/Low FAPs populations. We found that for both metrics, Sca-1 High FAPs increase markedly at 12 weeks post-denervation compared to the contralateral control and are sustained until 14 weeks, while Sca-1 Med/Low FAPs peak at 5 weeks and, by 12 and 14 weeks decrease back to baseline levels. This data is reported in the new Figure 6A,B. In addition, the proportion of Sca-1 High to Sca-1 Med/Low FAPs increases substantially at 12 weeks, with Sca-1 High FAPs comprising approximately half of the total FAPs population (Figure 6C). In the discussion (revised manuscript Discussion paragraph 5) we delineate the previously reported differential propensities for the induction of adipogenesis by the Sca-1 High vs Sca-1 Med/Low populations, and how this is relevant to the fibro-fatty degradation of long term denervated muscle.

(*C15). Major - The massive reduction in the frequencies of the sca-1-negVCAM-1-pos myogenic subset within the time period of 12 weeks is beautifully and clearly demonstrated! However, it is also clearly seen that the sca-1+VCAM-1+ double positive subset markedly declines within 12 weeks post denervation. What is this subset and why was it excluded from the set of experiments? Does its frequency changes in the CONTRA muscle at different time points after injury (when calculated out of total CD31-CD45- cells)? These data should be presented and at least discussed.*
(R15). We have now completed an analysis of this "double positive" population, and show the Sca-1+/VCAM-1+ cells are a mixture of FAPs and MPs.  As suggested by another

reviewer, we co-immunostained our freshly sorted cell populations (immediately spun onto coverslips after isolation) with the alternative FAPs marker PDGFRα and Pax7 (satellite cell marker) to address cell sort purity of the FAPs and MPs. While we found all FAPs stained solely for PDGFRα and MPs stained solely for Pax7 confirming their purity, the Sca-1+/VCAM-1+ cells all stained for either PDGFRα or Pax7, suggesting the double positive population is a mix of FAPs and MPs (Supplementary Fig 3A). We confirmed this by inducing the differentiation of adipocytes, fibroblasts and myotubes from the double positive population grown in culture over 12 days (Supplementary Fig 3 B,C).

Others (Malecova et al 2018) have reported a sub-population of VCAM-1 expressing FAPs that is nearly absent in healthy muscle, but transiently increased with acute inflammation, and their persistence is associated with chronic muscle inflammation, cycling regenerative attempts and fibrosis (in mdx mice). Sca-1 expression on a very small proportion of MPs in healthy muscle, with a transient increase following injury during myoblast proliferation, has also been reported (Kafadar et al 2009). We were unable to separate this double population at sort further using our third marker – the self-conjugated ITGA7 antibody – because of the interaction between Sca-1 and ITGA7 primary antibodies.

We evaluated the changes in the expression of the double positive population over time (Supplementary Figure 3D,E,). The population in the contralateral limb is stable over 14 weeks, but the changes seen with early denervation vary depending on the quantification method used – assessing cell frequencies demonstrates a continual fall from baseline, but assessing cells counts/gm of muscle shows a temporary small increase. However both methods reveal the double positive population falls back to, or below baseline levels by 12 weeks.

We discuss these findings in detail and their implications for use of the protocol in the revised Discussion paragraph 6.

*(C16). Induced fibrogenic, adipogenic and myogenic differentiation of sca-1+VCAM-1- and MP cells should be tested in vitro and presented as well.*

(R16). As noted prior, these experiments have been done. All cell subsets have been cultured in fibrogenic, adipogenic and myogenic differentiation media (new Figure 4 and Supplementary Figures 2 and 3).

Discussion

(*C17). Which other cell markers have been previously used (also in human and mouse) for isolation of equivalent cell subsets and why sca-1 was chosen? This should be discussed. The features of sca-1+ stromal cells should be more clearly defined other than simply FAPs and the authors should relate also to the existence of a third cell subset: sca-1+VCAM-1+.*

(R17). As previously noted in comment 2 (C2) above, we now address the common use of Sca-1 or PDGFRα as a positive selection marker for FAPs in the manuscript introduction and discussion. In addition we delineate the technical reasons behind the choice of Sca-1 over PDGFRα. We have also completed new experiments to demonstrate that our FAPs sorted with Sca-1 as the positive selection marker, all stain positive for PDGFRα (new Figure 4A),

further confirming their purity and identity. Lastly, as noted above (comment 15) we identified the sca-1+/VCAM-1+ to be a mixture of FAPs and MPs and discuss this phenotypic heterogeneity in the manuscript discussion (paragraph 6).

References
*(C18). Major - The list of references should be updated and introduce most recent publications related to the field study. In its current form it appears outdated.*
(R18). We have amended the reference list, included the publications cited by the reviewer and added additional more recent studies.

**Reviewer #4:**
*Manuscript Summary:*
*The authors present a FAPS protocol for the simultaneous isolation of FAPs and MPs from rat skeletal muscle. While such protocols are standard in the mouse, in the rat to my knowledge isolation of FAPs is not standard. As the rat is a valuable model for musculoskeletal disease, this protocol adds to the toolbox for this system.*

*Major Concerns:*
*(C1). The authors use immunolabeling of cells for markers of terminal differentiation to fibroblasts, adipocytes and myofibers that have been cultured for 7-10 days to assess specificity of the isolation. I consider it mandatory to demonstrate purity of both populations to be controlled upon isolation. For FAPs, PDGFRalpha staining should be employed (which is, besides Sca-1, the second commonly accepted FAP marker), for MPs combined labeling for myogenic progenitor markers as Pax7 and Myf5 or MyoD should be used.*
(R1). Thank-you for this suggestion. We have performed co-immunostaining for PDGFRα and Pax7 on FAPs (Lin-/Sca-1+/VCAM-1-) and MPs (Lin-/Sca-1-/VCAM-1+) immediately post-sorting, for initial validation of sort purity. We found that FAPs expressed PDGFRα with an absence of Pax7 positive cells, while MPs expressed Pax7 localized to the nucleus with no PDGFRα cross-contaminating cells (Figure 4A). Given that we were able to complete immunostaining on cells within minutes of cell sorting, we speculated all MPs would not have time to initiate *in vitro* differentiation transition and would retain strong expression of Pax7. This was confirmed and thus Myf5 and MyoD staining was not conducted.

(C2). Also, FAPs should be subjected to myogenic differentiation conditions, and MPs should be subjected to adipogenic conditions to rule out contaminations. Also, staining for myogenic progenitor markers should be used here.
(R2). In line with Reviewer #3's comment, we have incubated FAPs and MPs in myogenic, fibrogenic and adipogenic differentiation media. We found no cross-contamination of FAPs in MP cultures subjected to adipogenic and fibrogenic conditions, and no MPs in FAP cultures subjected to myogenic conditions (Supplementary Figure 2).

*(C3). P16: "In contrast, we did not observe increased VCAM-1expression in Sca-1 positive cells in the 2-to-14-week span following gastrocnemius denervation." Where is this shown?*
(R3). This was originally shown in Figure 5C. We were specifically referring to the double positive population within Sca-1::APC vs VCAM-1::PE plots of contralateral healthy and denervated samples. In this revision we have characterized the double positive population extensively, and found it to be a mix of FAPs and MPs. Heterogeneity in both of these progenitor lines has been previously reported, with small percentages of FAPs and MPs in healthy muscle co-expressing Sca-1 and VCAM-1. This is now discussed in detail (revised manuscript Discussion paragraph 6) and a new Supplementary Fig 3, including implications for the use of the protocol.

Minor Concerns:
*(C1). "Hoescht" dye should be "Hoechst"*
(R1). Spelling has been corrected.

*(C2). Fig. 5F, G are mentioned in the text before Fig. 5E; maybe rearrange the figure?*
(R2). We have added additional experiments to the revised manuscript that necessitated rearrangement of the figures. Figure 5F and G, previously detailing picrosirius red staining of denervated muscle cross-sections and accompanying quantification, are now Figure 5C-F.

**Supplementary File**

**Reagent Recipes**

*i) DMEM + 1% P/S*

- 99% DMEM
- 1% Penicillin-streptomycin

*ii) DMEM + 10% FBS & 1% P/S (For Flow Cytometry Protocol)*

- 89% DMEM
- 10% FBS
- 1% Penicillin-streptomycin

*iii) FAPs Growth Media*

- 90% Base Media:
  - 79% DMEM
  - 20% FBS
  - 1% Penicillin-Streptomycin
- 10% Heat-inactivated Horse Serum
- 2.5 ng/mL basic Fibroblast Growth Factor (bFGF)

*iv) FAPs Adipogenic Differentiation Media*

- 78% DMEM
- 20% FBS
- 1% Penicillin/Streptomycin
- 1.25 µM Dexamethasone (stock concentration 5 mg/mL) stock solution
- 0.5 mM IBMX (stock concentration 10 mg/mL)
- 5 µM Troglitazone stock solution (stock concentration 1 mg/mL)
- 1 µg/mL Humulin R (stock concentration 1 mM)

*v) FAPs Fibrogenic Differentiation Media*

- 89% DMEM
- 10% FBS
- 1% Penicillin-Streptomycin
- 1 ng/mL TGF-β1

*vi) MPs Growth Media*

- 40% DMEM
- 20% FBS
- 39% Ham's F10 Base Media
- 1% Penicillin-Streptomycin

*vii) MPs Differentiation Media*

- 97% DMEM
- 2% Horse Serum

- 1% Penicillin-Streptomycin
- 1 µg/mL Humulin R (stock concentration 1 mM)

*viii) Red Blood Cell (RBC) Lysis Buffer (10X)*

- Recipe (100 mL):
    - 9 g Ammonium Chloride ($NH_4Cl$) [Final Concentration 155 mM]
    - 1 g Potassium Bicarbonate ($KHCO_3$) [Final Concentration 10 mM]
    - 37 mg EDTA [Final Concentration 0.1 mM]
    - 100 mL double distilled water ($ddH_2O$)
- Sterilize through 0.2 µm filter
- Store at 4 °C; should be re-made every 2-3 weeks
- Dilute to 1X using sterile $ddH_2O$ on day of prep as needed

*ix) Wash Buffer*

- Recipe (100 mL):
    - 90 mL 1X PBS
    - 10 mL 20 mM EDTA in 1X PBS
    - 10 mL 250 mM HEPES in 1X PBS
    - 2 mL FBS
- Store at 4 °C

*x) Oil Red O Master Stock*

- Recipe (200 mL):
    - 0.7 g Oil Red O powder
    - 200 mL Isopropanol
- Stir overnight at room temperature, then process through 0.2 µm filter and store at 4 °C

*xi) Tissue IF Blocking Solution*

- Recipe (100 mL):
    - 2 g BSA
    - 5 mL FBS
    - 5 mL Goat Serum
    - 200 µL Triton-X-100
    - 0.1 g Sodium Azide
    - 200 mL 1X PBS

**Antibody Self-Conjugation**

*i) Sca-1 conjugation to APC (Derived from Biotium commercial kit protocol)*

Before commencing protocol, make sure to spin down all reagents to collect all powders/solutions to the bottom of the tube. Beware that some reagents (e.g. Linking Agent) are provided in very small quantities not visible to the naked eye.

1. Add 100 µL (100 µg) Sca-1 primary antibody to provided vial of Linking Agent. Pipette solution up and down 10 times to mix completely, then incubate the solution at room temperature for 30 mins.

2. Add the solution to the membrane of the spin column provided with the conjugation kit. Take care as to not touch the pipette tip to the membrane of the spin column. Then add 200 µL 1X PBS to the membrane.

3. Centrifuge the vial at 14,000 x g, 20 °C for 5 minutes to bind the linked antibody to the spin column membrane. Discard the flow-through in the tube.

4. Add 200 µL 1X PBS to the spin column and centrifuge again at 14,000 x g, 20 °C for 5 minutes. Discard the flow-through in the tube.

5. Resuspend the bound antibody to a concentration of 1 µg/µL by adding 100 µL of 1X PBS to the membrane. Gently pipet up and down 10 times over the surface of the membrane to recover the linked antibody.

   NOTE: The linked antibody cannot be seen with the naked eye, so the recovery of antibody will not be visible.

6. Transfer the entire recovered antibody solution to the provided vial containing lyophilized APC powder. Gently but thoroughly pipette up and down and on the sides of the vial, as well as gently vortex and spin to completely mix the antibody with APC.

7. Incubate the conjugate at room temperature in the dark for 3-4 hours.

8. Add 200 µL of provided conjugate storage buffer, bringing the final antibody concentration to 0.33 µg/µL with a total volume of 300 µL. Aliquot and store at -20 °C.

*ii) ITGA7 conjugation to PE-Cy7 (Derived from Abcam commercial kit protocol)*
Before commencing protocol, make sure to incubate all reagents for at least 15 minutes to warm up to room temperature. Furthermore, gently vortex and spin down all powders/solutions to ensure collection at the bottom of the tube.

1. Pipet 60 µL of ITGA7 primary antibody (1 µg/µL) to a fresh 1.5 mL microcentrifuge tube. Add 6 µL of provided modifier reagent to the tube and mix gently by pipetting up and down 5-10 times.

2. Pipette modified antibody solution to the provided vial of lyophilized PE-Cy7 powder. Re-suspend solution by gently pipetting up and down once or twice, making sure to collect all of the powder off of the sides of the vial.
3. Incubate vial for 3 hours in the dark at room temperature.

NOTE: Incubations can be left overnight with no negative effects on conjugation efficiency.

4. Add 6 µL of provided quencher reagent to quench any un-bound free PE-Cy7 dye. Mix gently by pipetting up and down 3 times and incubate at room temperature in the dark for 30 mins.

5. Aliquot the conjugated antibody and store at 4 °C.

   NOTE: Conjugated antibodies retain effectiveness for up to 3 months when stored at 4 °C. If storage at -20 °C is desired, add a cryoprotectant (e.g. 50% glycerol).