Chapter 17

Identification, Isolation, and Characterization of Mesenchymal Progenitors in Mouse and Human Skeletal Muscle

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Abstract

Mesenchymal progenitors residing in the muscle interstitial space contribute to pathogeneses such as fat infiltration and fibrosis. Because fat infiltration and fibrosis are hallmarks of diseased muscle, it is important to establish an accurate and reproducible method for isolating mesenchymal progenitors for research on muscle diseases. In this chapter, we describe methods based on fluorescence-activated cell sorting (FACS) to purify mesenchymal progenitors from mouse and human skeletal muscle using the most reliable marker for mesenchymal progenitors, PDGFRα. These methods allow concurrent isolation of the muscle stem cells called satellite cells. The quality of isolated mesenchymal progenitors is confirmed by their remarkable adipogenic potential without myogenic capacity, while purified satellite cells possess robust myogenic activity with no adipogenic potential. Simultaneous isolation of both mesenchymal progenitors and satellite cells from mouse and human tissues provides a powerful platform for studying skeletal muscle regeneration and diseases.

Key words Skeletal muscle, Mesenchymal progenitors, Satellite cells, Cell isolation, FACS

1 Introduction

Skeletal muscle has remarkable regenerative capacity that depends on a stem cell population called satellite cells [1]. Satellite cells are mononucleated cells residing between the plasma membrane of myofibers and the basal lamina [2]. Their high myogenic potential is essential to adult muscle regeneration and cannot be replaced by other cell types [3–5]. On the other hand, mesenchymal progenitors reside in the muscle interstitium, and therefore represent a cell population distinct from satellite cells [6]. These cells lack myogenic capacity but possess high potential to differentiate into mesenchymal lineages such as adipose and osteogenic cells [6, 7]. Mesenchymal progenitors are pathologically important because they directly contribute to fat infiltration and fibrosis [6, 8–10], which are commonly observed in diseased muscle. Because skeletal muscle cells dissociated by enzymatic digestion

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contain both mesenchymal progenitors and satellite cells, it is important to distinguish mesenchymal progenitors from satellite cells. Identifying target cells by specific markers and purifying them by fluorescence-activated cell sorting (FACS) represent the most effective and reliable method to achieve this. As a marker of mesenchymal progenitors, PDGFRα is considered the best because it is highly specific and can be used for both mouse and human tissues [11].

In the following sections, we describe detailed methods for the purification of mesenchymal progenitors from mouse and human skeletal muscle using PDGFRα as a positive marker. These methods enable simultaneous isolation of both mesenchymal progenitors and satellite cells. Methods to assess the quality of isolated cells by inducing adipogenic differentiation of mesenchymal progenitors or myogenic differentiation of satellite cells are also included.

2 Materials

2.1 Dissociating Cells from Skeletal Muscle

1. Mice: 8- to 10-week-old female mice (*see* **Note 1**).
2. Human muscle tissues (*see* **Note 2**).
3. Forceps and scissors. For trimming and mincing muscle tissues, fine-tipped forceps and curved scissors are recommended.
4. Sterile 60 mm tissue culture dishes.
5. Sterile 5, 10, and 25 ml pipettes.
6. PBS without Ca^{2+} and Mg^{2+} (sterile).
7. Dissection microscope.
8. Hank's balanced saline solution (HBSS) (sterile).
9. Collagenase Type II (Worthington, cat#: CLSS2).
10. Sterile 5 or 10 ml syringe.
11. Sterile 0.22-μm PVDF membrane syringe-driven filter unit.
12. A 20 ml beaker.
13. Magnetic stirrer and stir bar.
14. Tissue culture incubator (humidified, 5% CO_2, ambient O_2, 37 °C).
15. An 18-gauge needle.
16. Sterile 40 μm cell strainers.
17. Sterile 100 μm cell strainers.
18. Sterile 15 and 50 ml conical tubes.
19. Benchtop centrifuge.
20. Hypotonic solution: prepare 0.83% NH_4Cl and 0.17 M Tris HCl (pH 7.65) separately, and autoclave them separately. Mix at the ratio of 9:1.

21. Sterile 200 and 1000 μl pipette tips.
22. Hemocytometer.
23. Washing buffer: PBS supplemented with 2.5% fetal bovine serum (FBS).
24. Growth medium (GM): high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% FBS, 2 mM L-glutamine, 1% penicillin–streptomycin, and 2.5 ng/ml basic fibroblast growth factor (bFGF).
25. Sterile 60 mm dishes coated with collagen I.
26. Tissue culture incubator (humidified, 5% CO_2, 3% O_2, 37 °C).

2.2 Purification of Mesenchymal Progenitors and Satellite Cells from Dissociated Cells by FACS

1. Sterile phosphate-buffered saline (PBS) without Ca2+ and Mg2+.
2. Sterile 0.05% trypsin–EDTA.
3. Trypsin stop solution: PBS supplemented with 10% FBS.
4. Washing buffer, as above.
5. Sterile 1.5 ml microcentrifuge tubes.
6. Benchtop centrifuge.
7. Sterile 10, 200, and 1000 μl pipette tips.
8. Antibodies and secondary reagents: Alexa Fluor 488-conjugated rat anti-mouse CD31 (BioLegend, clone: 390), Alexa Fluor 488-conjugated rat anti-mouse CD45 (BioLegend, clone: 30-F11), goat polyclonal anti-mouse PDGFRα (R&D, cat#: AF1062), biotinylated rat anti-mouse satellite cells ([12], clone: SM/C-2.6), biotinylated goat polyclonal anti-human PDGFRα (R&D, cat#: BAF322), PE-conjugated mouse anti-human CD56 (Miltenyi Biotec, clone: AF12-7H3), PE-conjugated donkey polyclonal anti-goat IgG (Jackson ImmunoResearch, cat#: 705-116-147), streptavidin-PE/Cy5 (BioLegend), Alexa Fluor 488-conjugated rat isotype control (BioLegend), and PE-conjugated mouse isotype control (Miltenyi Biotec).
9. Sterile 40 μm cell strainers.
10. Sterile 50 ml conical tubes.
11. 5 ml round-bottom FACS tubes.
12. Propidium iodide (PI) solution.
13. Cell sorter.

2.3 Culture and Differentiation of Mesenchymal Progenitors and Satellite Cells

1. GM, as above.
2. Adipogenic induction medium (AdipoIM): DMEM supplemented with 10% FBS, 0.5 mM IBMX, 0.25 μM dexamethasone, 10 μg/ml insulin, 2 mM L-glutamine, and 1% penicillin–streptomycin.

3. Adipogenic maintenance medium (AdipoMM): DMEM supplemented with 10% FBS, 10 μg/ml insulin, 2 mM L-glutamine, and 1% penicillin–streptomycin.
4. Differentiation medium (DM): DMEM supplemented with 5% horse serum, 2 mM L-glutamine, and 1% penicillin–streptomycin.
5. Benchtop centrifuge.
6. Sterile 200 and 1000 μl pipette tips.
7. Matrigel diluted to 1 mg/ml with DMEM.
8. 48-well cell culture plates.
9. Tissue culture incubator (humidified, 5% CO_2, 3% O_2, 37 °C; or humidified, 5% CO_2, ambient O_2, 37 °C).
10. Inverted phase-contrast microscope.

3 Methods

3.1 Dissociating Cells from Skeletal Muscle

The procedures for dissociating cells from mouse and human skeletal muscle are essentially identical. Therefore, we describe procedures for mouse unless otherwise specified.

1. Before processing muscle tissue, autoclave forceps and scissors. Place a stir bar in a 20 ml beaker, cover beaker with aluminum foil, and then autoclave them.
2. Weigh a 60 mm tissue culture dish containing PBS.
3. Excise hind limb muscles of mouse. Transfer excised mouse muscles (or human muscle tissues) to the 60 mm tissue culture dish containing PBS (*see* Fig. 1a) and then weigh dish.
4. Calculate tissue weight by subtracting weight in **step 2** from weight in **step 3**.
5. Carefully remove remaining nerves, blood vessels, tendons, and fat using fine-tipped forceps under a dissection microscope.

The following steps should be performed in a sterile laminar flow hood.

6. Transfer trimmed muscles into a new 60 mm tissue dish and mince them thoroughly using the curved scissors (*see* Fig. 1b).
7. Dissolve collagenase type II in HBSS to make 0.2% collagenase solution. 4 ml collagenase solution per g of tissue is required for digestion (*see* **Note 3**). Sterilize collagenase solution by forcing it through 0.22 μm syringe-driven filter unit into the autoclaved beaker.
8. Transfer minced muscles into the beaker containing collagenase solution with the stir bar. Cover the beaker with aluminum foil.
9. Place the beaker and magnetic stirrer in tissue culture incubator, and then digest muscles for 50 min (30 min for human)

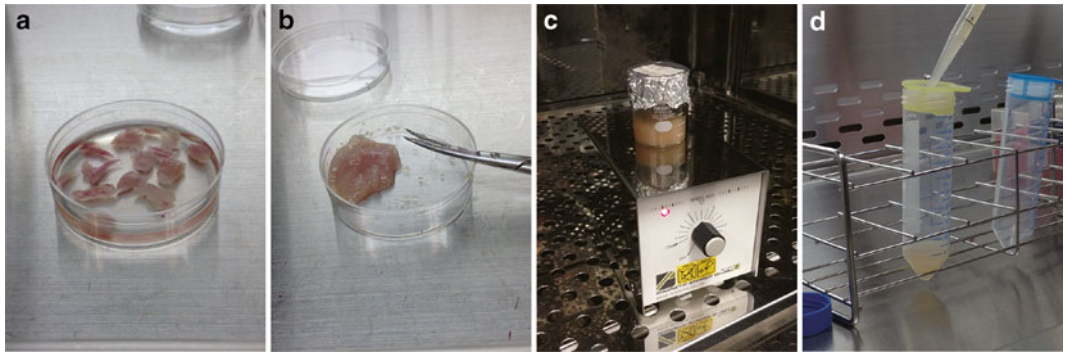

Fig. 1 Dissociating cells from skeletal muscle. (**a**) Excised muscles in PBS. (**b**) Minced muscle. (**c**) Digestion of muscle using magnetic stirrer. (**d**) Filtration of digested muscle slurry

at 37 °C with stirring by the magnetic stirrer (*see* Fig. 1c, *see* **Note 4**).

10. Pass digested muscles through the 18 gauge needle five to seven times using a sterile syringe.
11. Continue digestion for 20 min (15 min for human tissue) at 37 °C with stirring by the magnetic stirrer (*see* **Note 4**).
12. Add 10 ml PBS to digested slurry and mix thoroughly using 10 ml pipette.
13. Filter digested slurry through 100 μm cell strainer over a 50 ml conical tube (*see* Fig. 1d). Dilute digested slurry by washing cell strainer with PBS and adjust total volume to 25 ml/g of tissue (*see* **Note 5**).
14. Filter the slurry filtered in **step 13** through a 40 μm cell strainer on a new 50 ml conical tube. Dilute the slurry by washing cell strainer with PBS and adjust total volume to 50 ml/g of tissue (*see* **Note 5**).
15. Centrifuge cells for 5 min at 760 × *g*.
16. Resuspend the pellet in hypotonic solution. Use 2 ml hypotonic solution per gram of tissue. Transfer cells to a 15 ml conical tube and incubate for 1 min at room temperature to eliminate erythrocytes. Add at least 1 volume of PBS. Count cells using a hemocytometer.
17. Centrifuge cells for 5 min at 760 × *g*.
18. For mouse tissues, resuspend the pellet in washing buffer and adjust cell concentration to 1×10^7 cells/ml. Proceed to Subheading 3.2.
19. For human tissues, resuspend the pellet in 5 ml GM and culture on 60 mm collagen I-coated dish at 37 °C in 5% CO_2 and 3% O_2 (*see* **Note 6**). Maintain culture by changing GM every 3–4 days until cells reach 70–80% confluence.

3.2 Purification of Mesenchymal Progenitors and Satellite Cells from Dissociated Cells by FACS

3.2.1 Antibody Staining of Mouse Cells for FACS

1. Divide cells resuspended in washing buffer (**step 18** in Subheading 3.1) into five sterile 1.5 ml microcentrifuge tubes. Label these tubes "A" to "E": tube A for unstained control, tube B for Alexa Fluor 488 single-stained control, tube C for PE single-stained control, tube D for PE/Cy5 single-stained control, and tube E for triple-stained sample. Unstained or single-stained controls are used for compensation settings (*see* **Note 7**).
2. Add Alexa Fluor 488-conjugated rat isotype control (1:250) to tube A. Add Alexa Fluor 488-conjugated rat anti-mouse CD31 (1:250) and Alexa Fluor 488-conjugated rat anti-mouse CD45 (1:250) to tube B; add goat polyclonal anti-mouse PDGFRα (final conc. 2.5 μg/ml) to tube C; add biotinylated rat SM/C-2.6 (1:250) to tube D; and add Alexa Fluor 488-conjugated rat anti-mouse CD31, Alexa Fluor 488-conjugated rat anti-mouse CD45, goat polyclonal anti-mouse PDGFRα, and biotinylated rat SM/C-2.6 to tube E.
3. Incubate samples at 4 °C for 30 min in the dark.
4. Fill the tubes with washing buffer, and centrifuge cells for 5 min at 760 × *g*.
5. Resuspend the pellet in washing buffer and adjust cell concentration to 1×10^7 cells/ml.
6. Add PE-conjugated donkey polyclonal anti-goat IgG (1:200) and streptavidin-PE/Cy5 (1:200) to tube A. Add PE-conjugated donkey polyclonal anti-goat IgG to tube C, streptavidin-PE/Cy5 to tube D, and PE-conjugated donkey polyclonal anti-goat IgG and streptavidin-PE/Cy5 to tube E.
7. Incubate samples at 4 °C for 30 min in the dark.
8. Fill the tubes with washing buffer and centrifuge cells for 5 min at 760 × *g*.
9. Resuspend the pellet in washing buffer and adjust volume to 1 ml/tube.
10. Filter control and stained samples through 40-μm cell strainers placed on 50 ml conical tubes. Transfer filtered control and stained samples to 5 ml FACS round-bottom tubes.

3.2.2 Antibody Staining of Human Cells for FACS

1. Human cells at 70–80 % confluence (**step 19** in Subheading 3.1) are ready for FACS. Aspirate GM and wash cells with PBS.
2. Add 1 ml 0.05 % trypsin–EDTA per 60 mm dish and incubate cells for 3–5 min at 37 °C.
3. Add 1 ml trypsin stop solution and detach cells by gentle pipetting using a 1000 μl pipette tip. Transfer cells to a 15 ml conical tube. Count cells using a hemocytometer.
4. Centrifuge cells for 5 min at 430 × *g*.

5. Resuspend the pellet in washing buffer and adjust cell concentration to 5×10^6 cells/ml.
6. Divide cells resuspended in washing buffer into four sterile 1.5 ml microcentrifuge tubes. Label these tubes "A" to "D": tube A for the unstained control, tube B for PE single-stained control, tube C for PE/Cy5 single-stained control, and tube D for double-stained sample. Unstained and single-stained controls are used for compensation settings (*see* **Note 8**).
7. Add PE-conjugated mouse isotype control (1:20) to tube A. Add PE-conjugated mouse anti-human CD56 (1:20) to tube B, biotinylated goat polyclonal anti-human PDGFRα (final conc. 2.5 μg/ml) to tube C, and PE-conjugated mouse anti-human CD56 and biotinylated goat polyclonal anti-human PDGFRα to tube D.
8. Incubate samples for 30 min at 4 °C in the dark.
9. Fill up the tubes with washing buffer and centrifuge cells for 5 min at $430 \times g$.
10. Resuspend the pellet in washing buffer and adjust cell concentration to 5×10^6 cells/ml.
11. Add streptavidin-PE/Cy5 (1:200) to tubes A, C, and D.
12. Incubate samples for 30 min at 4 °C in the dark.
13. Fill the tubes with washing buffer and centrifuge cells for 5 min at $430 \times g$.
14. Resuspend the pellet in washing buffer and adjust volume to 1 ml/tube.
15. Filter control and stained samples through 40-μm cell strainer caps on 5 ml FACS round-bottom tubes.

3.2.3 Purification of Mesenchymal Progenitors and Satellite Cells from Mouse by FACS

Alexa Fluor 488, PE, and PE/Cy5 are excited by a 488 nm laser and detected by an appropriate filter set. Because the emission wavelength of PI excited by a 488 nm laser overlaps with that of PE/Cy5, PI should be excited by a UV laser and detected by a 620/29 bandpass filter. General settings of the cell sorter should follow the manufacturer's instructions. We describe here detailed procedures specific to purification of mesenchymal progenitors and satellite cells from skeletal muscle briefly.

1. Exclude debris on a forward scatter (FSC) vs. side scatter (SSC) graph.
2. Adjust detector voltage and compensation to optimal levels to clearly visualize negative and positive populations by analyzing unstained or single-stained controls (tubes A–D in Subheading 3.2.1).
3. Add 10 μl PI solution per 1×10^6 cells and mix gently. Exclude dead cells (PI-positive cells) on a PI vs. FSC or SSC graph.

4. Analyze triple-stained sample (tube E in Subheading 3.2.1). Gate for $CD31^-CD45^-$ cells as shown in Fig. 2a.
5. Display data of $CD31^-CD45^-$ cells on a PDGFRα-PE vs. SM/C-2.6-PE/Cy5 graph. Gate for $PDGFR\alpha^+SM/C\text{-}2.6^-$ cells (red gate) and $PDGFR\alpha^-SM/C\text{-}2.6^+$ cells (green gate) as shown in Fig. 2b.
6. Prepare collection tubes by adding 1 ml washing buffer to sterile 1.5 ml microcentrifuge tubes.
7. Sort $CD31^-CD45^-PDGFR\alpha^+SM/C\text{-}2.6^-$ cells as mesenchymal progenitors and $CD31^-CD45^-PDGFR\alpha^-SM/C\text{-}2.6^+$ cells as satellite cells (*see* **Note 9**).

3.2.4 Purification of Mesenchymal Progenitors and Satellite Cells from Human Tissue by FACS

1. Exclude debris on a forward scatter (FSC) vs. side scatter (SSC) graph.
2. Adjust detector voltage and compensation to optimal levels to clearly visualize negative and positive populations by analyzing unstained or single-stained controls (tubes A–C in Subheading 3.2.2).
3. Add 10 μl PI solution per 1×10^6 cells and mix gently. Exclude dead cells (PI-positive cells) on a PI vs. FSC or SSC graph.
4. Analyze double-stained sample (tube D in Subheading 3.2.2). Display data on a PDGFRα-PE/Cy5 vs. CD56-PE graph. Gate for $PDGFR\alpha^+CD56^-$ cells (red gate) and $PDGFR\alpha^-CD56^+$ cells (green gate) as shown in Fig. 3a.
5. Prepare collection tubes by adding 1 ml washing buffer to sterile 1.5 ml microcentrifuge tubes.
6. Sort $PDGFR\alpha^+CD56^-$ cells as mesenchymal progenitors and $PDGFR\alpha^-CD56^+$ cells as satellite cells (*see* **Note 10**).

3.3 Culture and Differentiation of Mesenchymal Progenitors and Satellite Cells

1. Add Matrigel to 48-well cell culture plates to coat the surface of the wells and then remove excess. Incubate the plates for 15 min at 37 °C. In a sterile laminar flow hood, uncover the plates and air dry.
2. Centrifuge sorted cells for 5 min at $760 \times g$ ($430 \times g$ for human cells) and resuspend the pellet in GM.
3. Add 1×10^4 cells to each well with 350 μl/well of the medium.
4. Incubate cells at 37 °C in 5% CO_2 and 3% O_2 for 3–4 days (*see* **Note 11**). After this growth period, mesenchymal progenitors and satellite cells reach ~70% confluence and are ready for induction of differentiation (*see* Figs. 2c, d, and 3b, c, *see* **Note 12**).
5. To induce adipogenic cells from mouse mesenchymal progenitors, remove GM and add adipoIM to the wells, and incubate at 37 °C in 5% CO_2 and ambient O_2 for 3 days. Then remove adipoIM and add adipoMM to the wells and incubate at 37 °C in 5% CO_2 and ambient O_2 for 3 days (*see* **Note 13**). After

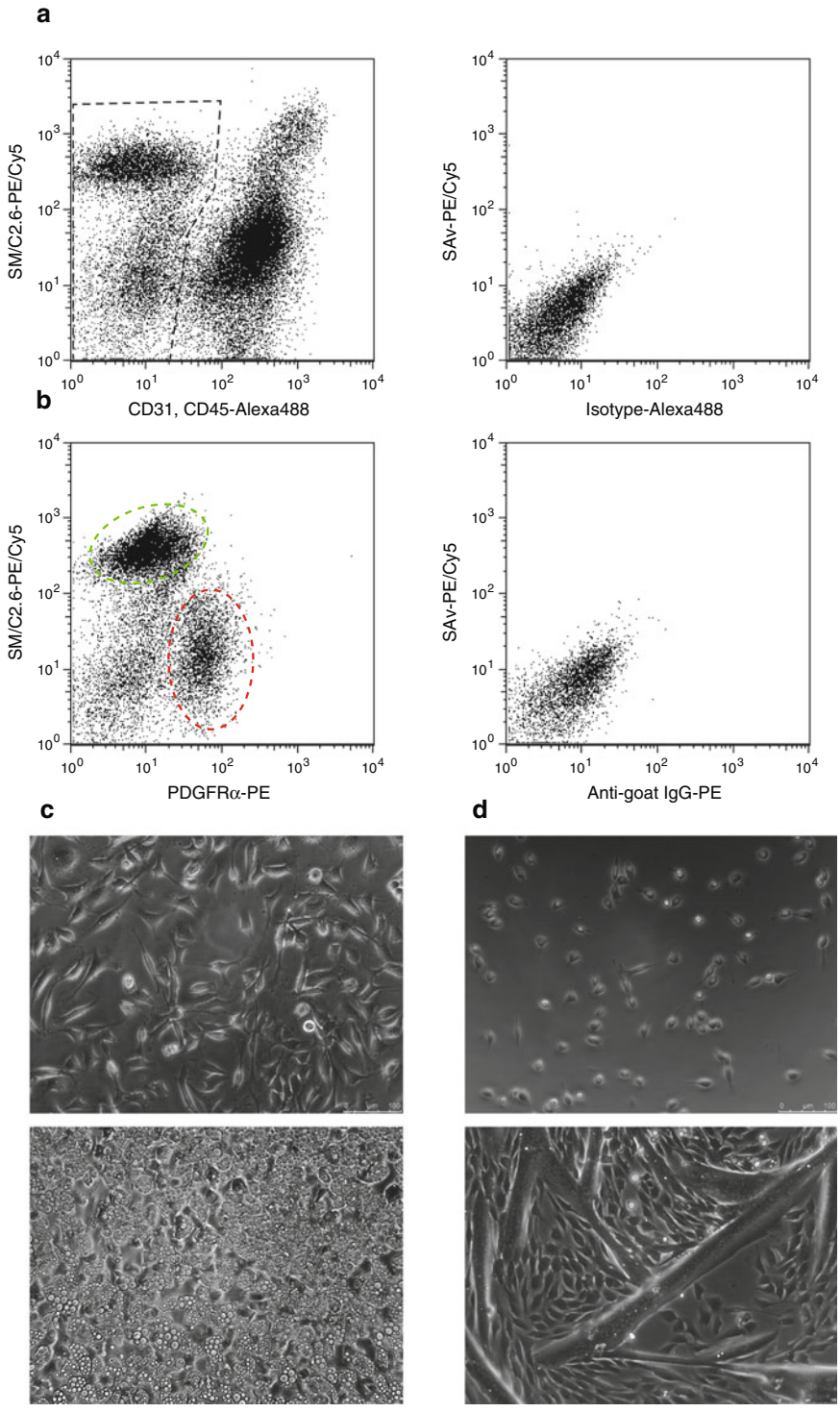

Fig. 2 Isolation and culture of mouse mesenchymal progenitors and satellite cells. (**a**) FACS dot plots showing the expression of CD31 and CD45 (*x*-axis) and SM/C-2.6 (*y*-axis). The unstained control is shown in the *right*. (**b**) FACS dot plots showing the expression of PDGFRα (*x*-axis) and SM/C-2.6 (*y*-axis) in $CD31^{-}CD45^{-}$ cells. The unstained control is shown in the *right*. (**c**) Morphology of cultured mouse mesenchymal progenitors in GM (*upper panel*) and after adipogenic differentiation (*lower panel*). (**d**) Morphology of cultured mouse satellite cells in GM (*upper panel*) and after myogenic differentiation (*lower panel*)

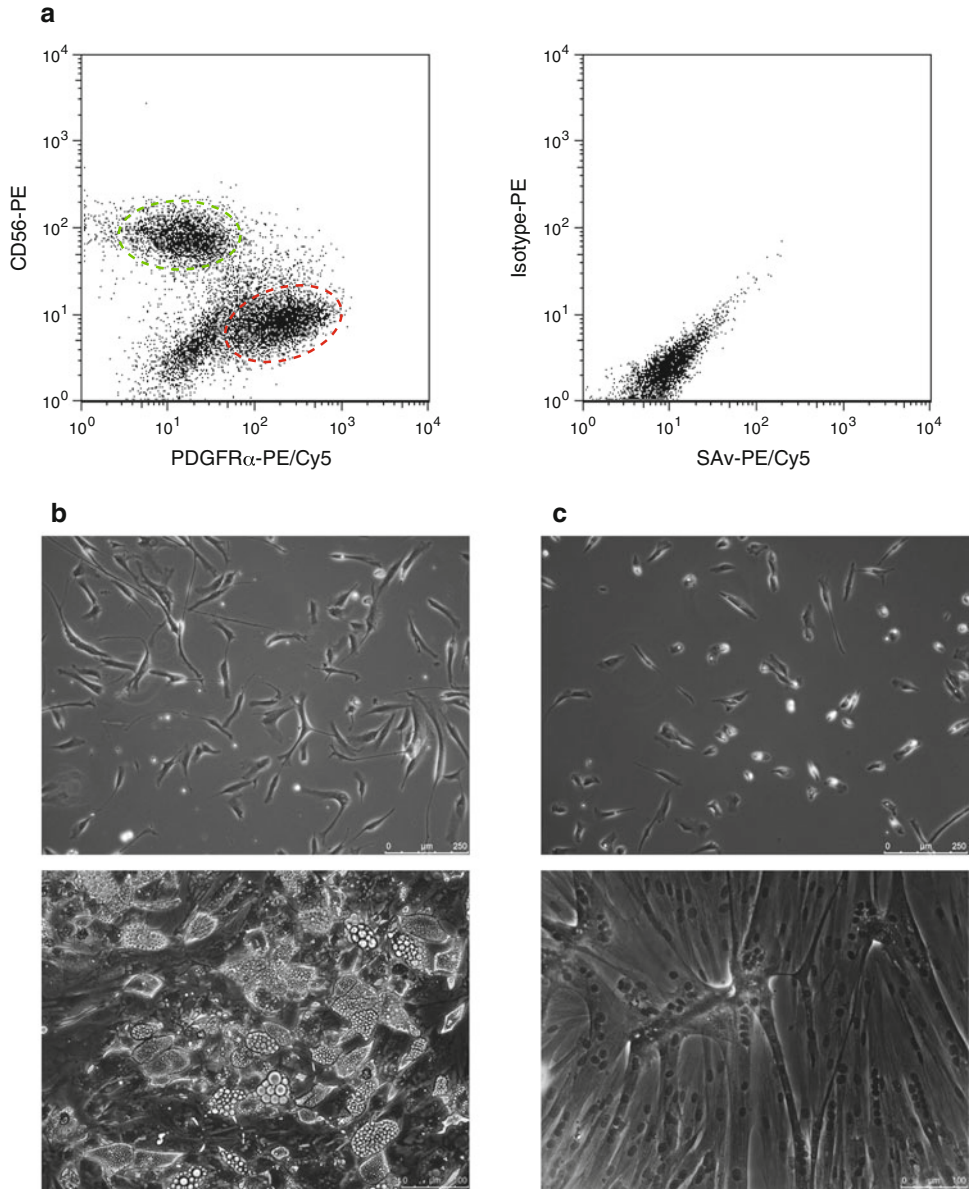

Fig. 3 Isolation and culture of human mesenchymal progenitors and satellite cells. (**a**) FACS dot plots showing the expression of PDGFRα (*x*-axis) and CD56 (*y*-axis). The unstained control is shown in the *right*. (**b**) Morphology of cultured human mesenchymal progenitors in GM (*upper panel*) and after adipogenic differentiation (*lower panel*). (**c**) Morphology of cultured human satellite cells in GM (*upper panel*) and after myogenic differentiation (*lower panel*)

adipogenic induction, mesenchymal progenitors differentiate into adipocytes containing lipid droplets that are readily revealed by an inverted phase-contrast microscope (*see* Fig. 2c).

6. To induce myogenic differentiation of mouse satellite cells, remove GM and add DM to the wells and incubate at 37 °C in

5 % CO_2 and ambient O_2 for 2–3 days (*see* **Note 13**). After myogenic differentiation, satellite cells develop into large myotubes that are readily revealed by inverted phase-contrast microscopy (*see* Fig. 2d).

7. To induce adipogenic cells from human mesenchymal progenitors, remove GM and add adipoIM to the wells and incubate at 37 °C in 5 % CO_2 and ambient O_2 for 3 days. Then remove adipoIM, add adipoMM to the wells, and incubate at 37 °C in 5 % CO_2 and ambient O_2 for 1 day (*see* **Note 13**). Repeat this induction and maintenance cycle three times. After adipogenic induction, mesenchymal progenitors differentiate into adipocytes containing lipid droplets that are readily revealed by inverted phase-contrast microscopy (*see* Fig. 3b).
8. For myogenic differentiation of human satellite cells, remove GM and add DM to the wells and incubate at 37 °C in 5 % CO_2 and ambient O_2 for 5 days (*see* **Note 13**). After myogenic differentiation, satellite cells develop into large myotubes that are readily revealed by an inverted phase-contrast microscope (*see* Fig. 3c).

4 Notes

1. Muscles of young female mice are digested more easily than those of male or older mice. We usually use C57BL/6, but we have confirmed that other strains such as BALB/c or ICR can be used as well.
2. Experiments using human tissues or cells must be approved by the institutional ethical review board.
3. Use at least 4 ml collagenase solution for efficient stirring even when tissue weight is less than 1 g.
4. Digestion time depends on sample condition. If male or older mice are used, longer digestion should be performed (e.g., we digest muscles of aged mice for 60 + 30 min).
5. Use one 50 ml conical tube with a 100-μm cell strainer and one 50 ml conical tube with a 40-μm cell strainer per gram of tissue (e.g., if starting tissue weight is 3 g, use three sets of conical tubes with cell strainers). If starting tissue weight is less than 1 g, use one conical tube with cell strainer and dilute digested slurry adjusting final volume to 50 ml.
6. Cell sorting by FACS requires a relatively large number of cells and therefore requires a large amount of starting tissue. However, a large amount of human tissue is difficult to obtain. We usually have less than 1 g of human muscle tissue. Therefore, it is necessary to expand cells before FACS in human experi-

ments. Note that a low oxygen concentration (3% O_2) is used for cell culture. This is critical for efficient expansion of human cells.

7. In mouse experiments, at least 5×10^5 cells should be used for the compensation setting (tubes A–D). All the remaining cells are used for the triple-stained sample (tube E).
8. In human experiments, at least 3×10^5 cells should be used for compensation setting (tubes A–C). All the remaining cells are used for the double-stained sample (tube D).
9. If the cell concentration is too high to analyze by FACS, dilute the sample with washing buffer as appropriate. It is important to use bright fluorophores for clear identification of mesenchymal progenitors and satellite cells. After careful optimization, we determined the use of goat anti-PDGFRα plus PE-anti-goat IgG (for identification of mesenchymal progenitors) and biotinylated-SM/C-2.6 plus streptavidin-PE/Cy5 (for identification of satellite cells) to be the most effective method. Cell yields by these methods are about 5×10^4 cells/young female mouse for mesenchymal progenitors and $1–1.5 \times 10^5$ cells/young female mouse for satellite cells.
10. Human cells are cultured for several days prior to FACS. Endothelial cells ($CD31^+$ cells) and hematopoietic cells ($CD45^+$ cells) are eliminated during this expansion period. If the cell concentration is too high to analyze by FACS, dilute the sample with washing buffer as appropriate. Cell yields from humans are highly variable among patients, as described in our paper [10].
11. A low oxygen concentration (3% O_2) is important for efficient growth of both mouse and human cells.
12. Mesenchymal progenitors are bigger than satellite cells and show a flattened morphology, while satellite cells show a compact morphology (*see* Figs. 2c, d, and 3b, c).
13. Although a low oxygen concentration is good for cell growth and expansion, cells differentiate better in the normoxic condition compared with the hypoxic condition. Thus, differentiation experiments should be performed under normoxic conditions.

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