Isolation of FAP Cells from Mouse Dystrophic Skeletal Muscle Using Fluorescence Activated Cell Sorting

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[Abstract] A population of muscle resident CD45$^-$, CD31$^-$ cells expressing the mesenchymal PDGF receptor alpha (PDGFRα) as well as Sca-1 was first isolated in healthy mouse muscles in Uezumi *et al*. (2010). In the same year, Joe *et al.* (2010) identified and purified fibro-adipogenic precursors (FAPs), cells located into the interstitial space between myofibers close to vessels, negative for CD45, CD31,α7-Integrin, but expressing CD34, Sca-1.

Both groups demonstrated that these cells are not myogenic *in vitro or in vivo*, but they are capable of differentiating *in vitro* towards both fibrogenic and adipogenic lineage (Uezumi *et al.*, 2011). Further marker analysis indicates that the two groups identified independently the same cell population (Natarajan *et al.*, 2010).

FAPs are an important source of fibrosis and adipogenesis in dystrophic skeletal muscle (Natarajan *et al.*, 2010; Cordani *et al.*, 2014). We have recently demonstrated that Nitric Oxide regulates FAP fate inhibiting *in vitro* their differentiation into adipocytes. In *mdx* mice, an animal model of DMD, fed with a diet containing the nitric oxide donating drug, Molsidomine, the number of PDGFRα^+ cells was reduced as well as the deposition of both skeletal muscle fat and connective tissues (Cordani *et al.*, 2014). Here we described a method to isolate in both wild type and in mdx dystrophic muscle pure population of FAPs by double selection for SCA-1 and PDGFRα positivity in absence of the satellite cell markers SM/C2.6 and α7integrin as well of the pan-lymphocytes marker CD45 or endothelial marker CD31.

Material and Reagents

1. 8-10 weeks old mice C57BL/6J wild-type mice (Charles River Laboratories International, http://www.criver.com) and mdx-4cv mice (B6Ros.Cg-Dmdmdx-4cv/J, crossed on C57/BL/6 background; Jackson ImmunoResearch Laboratories)
   Note: Animals were treated in accordance with European Community guidelines and with the approval of the Institutional Ethical Committee.
2. Collagenase II (Worthington Biochemical, catalog number: CLSS2)

3. Dulbecco's modified high glucose Eagle's medium (DMEM high glucose) (EuroClone, catalog number: ECB7501L)
4. 100 U/ml penicillin and 100 µg/ml streptomycin (EuroClone)
5. L-glutammine (EuroClone)
6. Recombinant human basic fibroblast growth factor (b-FGF) (Pepro Tech, catalog number: 100-18B)
7. Growth factor reduced BD Matrigel™ Matrix (BD Biosciences, catalog number: 356230)
8. Foetal Bovine Serum (FBS) (EuroClone)
9. Sterile phosphate buffered saline (PBS) w/o $Ca^{++}Mg^{++}$ (EuroClone)
10. Antibodies
    a. Anti-CD31-phycoerythrin/Cy7 (anti-CD31-PE/Cy7, clone 390) (eBioscience, catalog number: 25-0311)
    b. Anti-CD45-PECy7 (clone 30-F11) (eBioscience, catalog number: 15-0451)
    c. Anti-SM/C2.6-Biotin (kindly provided by Dr. Fukada) (Fukada *et al.*, 2004)
    d. Streptavidin-PE (BioLegend)
    e. Anti-α7-Integrin-PE (clone R2F2) (AbLab Laboratorio di Istologia e Citologia Patologica Veterinaria, catalog number: AB10RS24MW215)
    f. Anti-PDGFRα-allophycocyanin (APC, CD140a, clone APA5) (BioLegend, catalog number: 135907)
    g. Anti-LY-6A/E SCA-1-allophycocyanin/Cy7 (APC/Cy7, clone B7) (BD Biosciences, catalog number: 560654)
    h. 7-aminoactinomycin D (7-AAD) (Life Technologies, catalog number: A1310)
11. Growth medium (GM) (see Recipes)
12. Wash buffer (WB) (see Recipes)
13. Collagenase II solution (see Recipes)
14. Erythrocytes lysis buffer (see Recipes)
    Note: Use 1 ml of this solution for approximately 1 g of muscle.
15. Sorting buffer (see Recipes)
16. Matrigel solution (see Recipes)
17. b-FGF solution (see Recipes)

Equipment

1. Scissors and tweezers
2. Cell culture plastic dishes (Corning, Costar®)
3. Six multiwells (Corning, Costar®)
4. Centrifuge

5. 50 and 15 ml plastic tubes
6. 18G-10 ml syringes
7. 70 µm and 40 µm cell strainer caps (BD Biosciences)
8. Beckam Coulter Cell Sorter MoFlo™ XDP (Beckman Coulter, catalog number: ML99030)
9. Cell culture incubator at 37 °C and 5% CO_2
10. Microscope or cell counter

Procedure

1. Sacrifice the mice (C57BL/6 or MDX) by delivering increasing concentrations of CO_2 and remove hindlimb muscles.
2. Weigh the muscle mass.
3. Leave them in cold PBS-containing dish.
4. Remove visible tendon, adipose tissue and vessel.
5. Mince the muscles on new dish (not containing PBS) using a curved tip scissor for few minutes until the tissue appears like a mush.
6. Transfer the tissue into a 50 ml plastic tube and add 0.2% collagenase II in DMEM (serum-free). Volume to use is 2-4 ml of collagenase for 1 g of muscle weight: Usually each mouse allows obtaining 1-1.5 g of tissue. Put plastic tubes into a shaking thermostatic bath, at 37 °C for 1 h.
7. Separate undigested from digested material by centrifugation at 500 rpm. All the centrifugations are at room temperature. Supernatant (digested material) was removed, diluted with room temperature PBS (approximately 50 ml of PBS/1 g muscle) and go through a 18 G-10 ml syringe to create a single cell suspension (about 10 passages through the needle).
8. Add to the un-digesting muscle fresh collagenase solution (using the same volume of step 6) and leave in the shaking thermostatic bath, at 37 °C for additional 30 min. Then, repeat step 7. Discard eventually indigested material that may remain after the second centrifugation procedure.
9. Muscle slurries obtained by the two digestions were pulled and filtered through 70 µm cell strainer cap and subsequently through 40 µm cell strainer cap, using 50 ml syringes and 50 ml plastic tubes.
10. Centrifuge at 2,000 rpm for 10 min and remove supernatant using a pipette.
11. Eliminate blood red cells by re-suspending cell with 1-2 ml of erythrocyte lysis buffer.
12. Dilute erythrocyte lysis buffer by addiction of 50 ml of PBS containing 2% FBS and centrifuge 2,000 rpm for 10 min at room temperature.
13. After removing the supernatant, using a pipette, add 1 ml PBS containing 10% of FBS

and count the cells.

14. Centrifuge tubes 2,000 rpm for 10 min and incubate recovered cells for 30 min with anti-SM/C2.6-Biotin on ice. Use 1 µl of antibodies for 1 x 10^6 cells in 100 µl of PBS containing 2% of FBS.
15. Add 1 ml wash buffer and centrifuge 2,000 rpm for 5 min.
16. After removing the supernatant, incubate on ice for 30 min with Streptavidin-PE and with the other labeled antibodies [anti-CD31-PECy7 and anti-CD45-PECy7, anti-α7-Integrin-PE (for MDX mice), anti-PDGFRα-APC, anti-LY-6A/E SCA-1-APC/Cy7] and with 7-aminoactinomycin D. As in step 14, use 1 µl of antibodies for 1 x 10^6 cells in 100 µl of PBS containing 2% of FBS.
17. Add 50 ml of wash buffer and centrifuge 2,000 rpm for 5 min.
18. After removing the supernatant, add 2 ml of sorting buffer/mice.
19. To set up the instrument, sample of about 50-100,000 cells were in parallel individually stained with the each single antibody or with 7-aminoactinomycin (single staining for compensation) in the same conditions of samples to sort. A sample of 50-100,000 cells was also no incubated with any antibody (unstained control). After 30 min, add 1 ml of wash buffer then centrifuge (2,000 rpm for 5 min). Remove supernatant and add 200-300 µl of PBS containing 2% of FBS.
20. For cell sorter setting: Using unstained control, exclude debris and dead cells by forward scatter and side scatter and set basal fluorescence (Figure 1A). Then make 7-AAD gating using sample stained with 7-aminoactinomycin (gate 1, 7AAD negative cells and gate 2) to further eliminate dead ells.
21. Compensate fluorescence using the samples individually stained with each antibodies and then select in sample to sort FAP cells as $CD45^-/CD31^-$ (gate 3)/$smc2.6^-$ or $\alpha7integrin^-$ (gate 4)/$SCA\text{-}1^+$ and $PDGFR\alpha^+$ (gate 5) cells. To improve purity, we preferred to employ anti- smc2.6 for WT mice and anti-α7integrin for *mdx* mice.
22. At the end of sorting, plate FAP cells at 1 x 10^4 cells/cm^2 in multiwell plates previously coated with Matrigel and culture for 6 days in growth medium plus b-FGF in a humidified incubator (37 °C and 5% CO_2).

Representative data

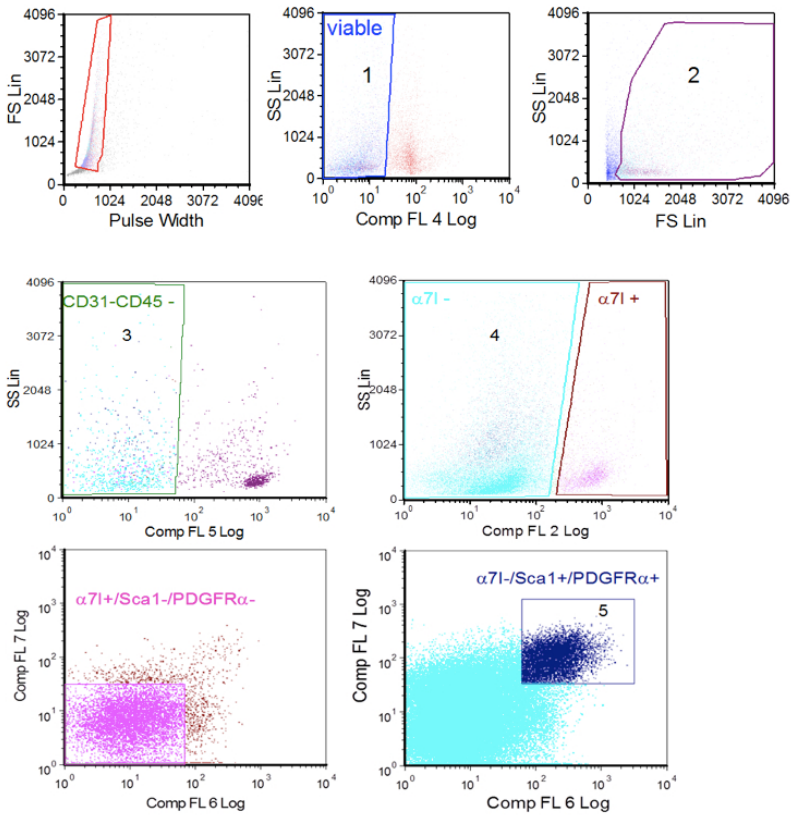

Figure 1. Cell sorter procedure. A. single cells gating; B. viable cells gating based on selection of 7ADD negative cells (1, left) and forward scatter/side scatter parametes (2, right); C. CD31 and CD45 negative cells gating (3); D. α7-integrin gating (4); E. satellite cells gating (Sca 1/PDGFRα negative cells from α7-integrin positive gate); F. FAP cells gatining (ScA 1/PDGFRα positive cells from the α7-intergrin negative gate (5).

Notes

1. FAP cells recovery range from 1 to 3% of total cells. Digestion seems to be the critical point.

Recipes

1. Growth medium (GM)
   Dulbecco's modified high glucose Eagle's medium (DMEM) supplemented with heat inactivated 20% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin plus 5 ng/ml of recombinant human basic fibroblast growth factor

2. Wash buffer (WB)
   PBS w/o Ca^{++} and Mg^{++} with heat inactivated 2% FBS
3. Collagenase II solution
   0.2% collagenase solution was prepared in DMEM, filtered on a 0.2 µm filter and immediately used.
4. Erythrocytes lysis buffer
   0.8% NH_4Cl in Tris-buffer (pH 8)
5. Sorting buffer
   PBS w/o Ca^{2+} and Mg^{2+} with 5% heat inactivated FBS
6. Matrigel solution
   Dilute Matrigel 1:100 in DMEM and use immediately after preparation for plate coating
   Left Matrigel working solution into the plate for 30 min before removing
   Matrigel must not dry
7. b-FGF solution
   Prepare 100 µg/ml stock solution aliquots and keep them at 20 °C
   In culture b-FGF concentration was 5 ng/ml
   After defrosting, b-FGF aliquot could remain at 4 °C and used within a week

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