Published in final edited form as:

Methods Mol Biol. 2013 ; 976: 53–65. doi:10.1007/978-1-62703-317-6_5.

Isolation of Muscle-Derived Stem/Progenitor Cells based on adhesion characteristics to collagen-coated surfaces

Mitra Lavasani^{1,2,*}, Aiping Lu^{1,2,*}, Seth D. Thompson¹, Paul D. Robbins^{2,3,4}, Johnny Huard^{1,2,4}, and Laura J. Niedernhofer^{3,4}

¹Stem Cell Research Center, 206 Bridgeside Point II, 450 Technology Drive, Pittsburgh, PA 15219

²Department of Orthopaedic Surgery, University of Pittsburgh School of Medicine, 3471 Fifth Avenue, Pittsburgh, PA 15213

³University of Pittsburgh Cancer Institute, 5117 Centre Avenue, Hillman Cancer Center 2.6, Pittsburgh, PA 15213

⁴Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, 523 Bridgeside Point II, 450 Technology Drive, Pittsburgh, PA 15219

Summary

Our lab developed and optimized a method, known as the modified pre-plate technique, to isolate stem/progenitor cells from skeletal muscle. This method separates different populations of myogenic cells based on their propensity to adhere to collagen I-coated surface. Based on their surface markers and stem-like properties, including self-renewal, multi-lineage differentiation, and ability to promote tissue regeneration, the last cell fraction or slowest to adhere to the collagen-coated surface (pre-plate 6; pp6) appear to be early, quiescent progenitor cells termed muscle-derived stem/progenitor cells (MDSPCs). The cell fractions preceding pp6 (pp1–5) are likely populations of more committed (differentiated) cells, including fibroblast- and myoblastlike cells. This technique may be used to isolate MDSPCs from skeletal muscle of humans or mice regardless of age, sex or disease state, although the yield of MDSPCs varies with age and health. MDSPCs can be used for regeneration of a variety of tissues including bone, articular cartilage, skeletal and cardiac muscle and nerve. MDSPCs are currently being tested in clinical trials for treatment of urinary incontinence and myocardial infarction. MDSPCs from young mice have also been demonstrated to extend lifespan and healthspan in mouse models of accelerated aging through an apparent paracrine/endocrine mechanism. Here we detail methods for isolation and characterization of MDSPCs.

Keywords

Pre-plate technique; muscle-derived stem/progenitor cells; muscle; adult stem cells; regenerative medicine

Address correspondence to J.H. (jhuard@pitt.edu) Stem Cell Research Center, 206 Bridgeside Point II, 450 Technology Drive, Pittsburgh, PA 15219, Phone: (412) 648-2798, Fax: (412) 648-4066, or L.J.N. (niedlx@upmc.edu), 5117 Centre Avenue, Hillman Cancer Center, 2.6, Pittsburgh, PA 15213, Phone: (412) 623-7763, Fax: (412) 623-7761.

*These authors contributed equally.

1. Introduction

Adult stem cells hold great promise for regenerative medicine. Stem cells isolated from adult organisms have significant advantages over embryonic and fetal stem cells as therapeutic modalities. First, adult stem cells by-pass the substantial regulatory burden that accompanies the use and study of cells isolated from perinatal organisms (1–3). Second, adult stem cells offer the possibility of autologous therapy. Using cells isolated from a patient to treat only that patient eliminates the risk of an immune rejection that could attenuate the therapeutic benefit or worse cause significant side effects (4). In addition, autologous approaches for cell-based therapies will accelerate the path to the clinic (5–7).

Adult stem cells have been isolated from the bone marrow (8, 9), brain (10, 11), muscle (12–14), fat (15), skin (16) and gut (17–19). Muscle-derived stem cells offer several advantages over other adult stem cell populations for therapeutic applications. First, skeletal muscle is relatively easy to access, unlike, for example, brain and bone marrow. Second, a relatively small amount of tissue is necessary to isolate a sufficient number of stem cells for treatment purposes (4). Third, functional MDSPCs can be expanded substantially *ex vivo* (20). This minimizes the amount of muscle that must be excised from a patient, but also offers the possibility of treating a patient multiple times after a single procedure to isolate stem cells. Fourth, there is evidence that MDSPCs isolated from older organisms, which have lost their potency, can be rejuvenated to behave more like MDSPCs isolated from young adults (21). This means that even the elderly may be eligible for autologous therapy. Fifth, it is established that MDSPCs can be transduced with retroviral vectors expressing novel genes while maintaining their stem-like properties (22–30). MDSP-like cells isolated from humans show high regenerative potential in skeletal and cardiac muscle (31, 32). Thus MDSPCs may potentially be used to treat inherited diseases of muscle degeneration, for example muscular dystrophies. MDSPCs can differentiate into myogenic, osteogenic, chondrogenic, adipogenic, neural, endothelial, and hematopoietic cells (13, 33–45). Therefore MDSPCs may be useful for treating degenerative diseases of multiple organ systems. Finally, MDSPCs appear to elicit a therapeutic benefit, at least in part, by secreting factors that promote host rejuvenation (21). Thus therapeutic benefits may be achieved with fewer cells.

To date, MDSPCs have been used to improve bone healing (26, 35, 46), cartilage repair (36, 47), cardiac ischemia (48, 49), urinary incontinence (7, 38, 50–53), aging-related degenerative changes (21), and muscular injury including muscular dystrophy (13, 34, 54). Although MDSPCs are not a well-defined, homogenous cell type, their tremendous potential for treating a wide spectrum of traumatic and degenerative changes, supports the continued development and analysis of MDSPCs. Herein is described in detail the methods necessary for the isolation and minimal characterization of MDSPCs.

2. Materials

2.1 Reagents

1. Hank's Buffered Salt Solution **plus** Calcium Chloride and Magnesium Chloride (HBSS; Invitrogen, Cat. #24020-117, *See Note 1*)
2. Collagenase type XI (Sigma-Aldrich, Cat. #C7657)
3. Dispase (Invitrogen, Cat. #17105-041)
4. Trypsin-EDTA (Invitrogen, Cat. #15400-054)

¹Using HBSS with Ca²⁺ and Mg²⁺ reduces the enzymatic activity of trypsin. But it is effective for gently releasing stem/progenitor cells from plates.

5. Dulbecco's Modified Eagle Medium (DMEM, high glucose; Invitrogen, Cat. #11995-073)
6. Fetal Bovine Serum (FBS; Invitrogen, Cat. #10437-028)
7. Horse Serum (HS; Invitrogen, Cat. #26050-088)
8. Chick Embryo Extract (CEE; Accurate Chemical Co. Cat. # CE650T-10) [*See Note 2*]
9. Penicillin/Streptomycin (P/S, Invitrogen, Cat. #15140-122)
10. Collagen, type I (Sigma-Aldrich, Cat. #C9791)
11. Dimethyl sulfoxide (DMSO, Sigma, Cat. #D-2650)

2.2 Laboratory supplies

1. Tubes: 15 mL and 50 mL polypropylene (e.g. BD, Cat. #352097 and #352098)
2. Filters: disposable sterile 0.22 μ m pore size and 500 mL sterile filter system (e.g. Corning, Cat. #430769)
3. Petri dishes: 35 mm and 65 mm (e.g. BD, Cat. #351008)
4. Cell strainer: disposable 70 μ m pore size (e.g. BD, Cat. #352350)
5. Flasks: 25 cm² (T-25) and 75 cm² (T-75) (e.g. BD, Cat. #353109 and #353136)
6. Cryovials (1.5 mL, e.g. Nalgene, Cat. #5000-1020, *See Note 3*)
7. "Mr. Frosty" freezing container (Nalgene, Cat. #5100-0001) or empty 15 mL Styrofoam boxes

2.3. Required equipment

1. Sterile surgical equipments (e.g. forceps, scissors)
2. Laminar flow tissue culture hood (e.g. The Baker Company, Model #SG 403)
3. Incubator to maintain 37°C, >95% humidity and an atmosphere of 5% CO₂ (e.g. HERAcell 150, Thermo Fisher, Cat. #51022393)
4. Refrigerated bench-top centrifuge (e.g. Legend RT, Thermo Fisher, Cat. #75004377)
5. Laboratory balance (e.g. Mettler Toledo, Model #PM4000)
6. Pipet-aid (e.g. Drummond Scientific, Cat. #4-000-101)
7. Adjustable micropipettes: 10 μ L, 200 μ L and 1000 μ L (e.g. Gilson, Model #P200N) with sterile disposable plastic pipette tips (e.g. Denville Scientific, Cat. #P1326-CPS)
8. Hemocytometer (e.g. Hausser 1475) or automatic cell counter (e.g. Invitrogen, Cat. #C10281)

²In our experience, CEE is critical for the isolation and maintenance of MDSPCs. CEE from different manufactures and even different lots produced by the same manufacturer has a profound effect on the quality of MDSPCs, including their capacity for proliferation and differentiation. The best results are obtained if the CEE is not pre-cleared by centrifugation or ultrafiltration.

³Ideally, MDSPCs should be stored submerged in liquid nitrogen. Note that this requires special cryovials that can withstand the pressure changes that occur when cells are thawed. It is also important not to overfill the cryovials to prevent the tubes from exploding during the thawing process.

9. Inverted light microscope with phase contrast capabilities and objectives of 5x, 10x, 20x magnification (e.g. Nikon, Model #TMS 215798)

3. Methods

3.1 Solution preparation

1. Tissue digestion solution: The working concentrations are 0.2% (wt/vol) collagenase Type-XI, 2.4 U/ mL dispase, and 0.1% (wt/vol) Trypsin-EDTA. Both solutions are prepared in HBSS and filtered using a 0.22- μ m sterile filter, aliquoted into 10 mL volume aliquots, and stored at -20°C . Solutions should be pre-warmed to 37°C before use.
2. Proliferation Medium (PM): Except for DMEM, all components of the PM should be preprepared and stored at -20°C . Pre-warm all the components to 37°C before use. To prepare 500 mL of PM, combine 392.5 mL DMEM, 50 mL FBS (10%), 50 mL HS (10%), and 5 mL P/S (1%) in a 500 sterile filter attached to the filter receiver. Add 2.5 mL CEE (0.5%) as the last 10 mL of solution is drawn through the sterile filter system, as it clogs the filter and retards the process. PM should be prepared fresh for each new culture of MDSPCs. Unused PM can be stored at 4°C . PM for a maximum of 4 weeks.
3. Freezing medium: 1:10 dilution of DMSO: FBS or PM (*See Note 4*).
4. Collagen coating solution:

Day 1—Under a laminar flow hood using sterile technique, add 0.1 g of collagen type I to a sterile 1-liter glass bottle with screw cap. In a sterile graduated cylinder, add 5.7 mL glacial acetic acid to 1 liter of double-distilled water. Add the mixture to the collagen by filtering the acid/water through a 500 mL bottle top sterilization filter. Stir with a magnetic stir bar overnight.

Day 2—Under a laminar flow hood using sterile technique, pour collagen type I into 50 mL sterile polystyrene centrifuge bottles and spin at $2,630 \times g$ for 20 min at 4°C . Transfer the supernatant to sterile (autoclaved) bottles while. Under a laminar flow hood, slowly add 50 mL of chloroform (caution: volatile) per 500 mL solution using a sterile glass funnel. The chloroform will form a layer under the collagen solution. **DO NOT SHAKE OR STIR.** Allow to sit overnight (can be extended to two days) at $2-8^{\circ}\text{C}$.

Day 3—Under a laminar flow hood using sterile technique, transfer the top aqueous layer (containing the collagen) to a new sterile 1-liter bottle and store at $2-8^{\circ}\text{C}$. Discard the bottom layer containing the chloroform according to your institution's hazardous waste guidelines.

3.2 Collagen coating flasks and plates

To coat the flasks or plates with Collagen Type-I, warm the solution in 37°C water bath. Add the recommended volume of collagen to the flasks/plates (*See Note 5*). Make sure the solution is evenly distributed by gently rocking them until the surface is covered. Repeat frequently over the next 2 hrs. Keep the flasks/plates open in culture hood with blower on for a minimum of 3–4 hrs, but ideally for 7 hrs. After this, pipette off the excess collagen solution to a flask label as “used collagen type-I”. This can be reused for future coating. Leave the flasks/plates partially open in a running laminar flow hood overnight to promote

⁴Viability of MDSPCs after thawing is dramatically improved if a 1:10 (v/v) ratio of DMSO:FBS is used rather than DMSO:media.

drying. Keep the flasks/plates in a laminar flow hood with the germicidal UV lamp on for 2 hrs. The next day, cap the flasks/plates, label, bag in the original wrapping and store at room temperature.

3.3 Isolating and digesting skeletal muscle from mice

1. Sterilize surgical tools.
2. Euthanize the animal by CO₂ asphyxiation.
3. Dissect out the skeletal muscle. For adult mice, use the hind limb muscles including gastrocnemius, soleus and quadriceps muscle (350–450 mg, also *See Note 6*). For young mice (1–7 days), the entire limb muscles should be used. Place the tissue from a single animal into a 15 mL tube containing sufficient HBSS to cover the tissue and store on ice.
4. Transfer one skeletal muscle at a time to a sterile 65 mm Petri dish and wash it three times with sterile HBSS to remove any debris that may have adhered to the tissue during the dissection.
5. Using forceps and scissors, dissect the muscle from other tissues including the bone, tendon, nerve, major blood vessels, fat, and connective tissue in a sterile 35 mm Petri dish.
6. Add 10 mL of sterile HBSS to a fresh Petri dish and begin the mechanical digestion of the trimmed muscle by mincing it into a coarse slurry using very sharp spring scissors.
7. Transfer the minced tissue slurry into a 15 mL conical tube using a 10 ml transfer pipette and centrifuge at $930 \times g$ at 2–8°C for 5 min.
8. Aspirate off the supernatant, resuspend the pellet with HBSS, and centrifuge again as described in step 4.
9. Aspirate off the supernatant and weigh the pellet (slurry) using a precise scale. Zero the scale using an empty 15 mL conical tube.
10. Begin the enzymatic digestion by adding 10 ml of pre-warmed 0.2% collagenase type XI digestion solution (approximately 1 mL per 0.1 g muscle pellet) in a 15 mL tube. Incubate at 37°C. Gently rock the tube by hand every 15 min. After 1 hr, centrifuge at $2,630 \times g$ for 5 min [*See Note 7*].

⁵Amount of collagen type I needed for coating of flasks/plates:

T25	2 ml
T75	3.5–4 ml
T175	7.5–8 ml
6-well plate	1 mL/well
12-well plate	0.5 mL/well
24-well plate	0.25 mL/well
96-well plate	50 µL/well

⁶For newborn animals, both the hindlimb and forelimb muscles are collected.

⁷Collagenase type XI and dispase digestion can be extended when a larger amount of muscle must be digested. But the cells are sensitive to prolonged exposure to Trypsin-EDTA.

11. Aspirate off the supernatant and resuspend the pellet/slurry in 10 mL pre-warmed dispase (2.4 units/mL). Incubate at 37°C for 45 minutes. Gently rock the tube by hand every 15 min. Centrifuge at $2,630 \times g$ for 5 min.
12. Aspirate off the supernatant and resuspend the viscous slurry in 0.1% Trypsin-EDTA solution diluted in HBSS. Incubate at 37°C for 30 min. Gently rock the tube by hand every 15 min. Centrifuge at $2,630 \times g$ for 5 min.
13. Aspirate out the supernatant and resuspend the cell pellet in 10 mL of PM.
14. Pipette the resuspended pellet through a 70 μ m cell strainer placed on top of a 50 mL sterile conical tube. Rinse the strainer with PM to collect all of the cells.

3.3 Pre-plating steps (Figure 1)

1. Plate the cells from 1 animal/tube on 1 collagen type I-coated T-25 flask and incubate at 37 °C in a humidified, 5% CO₂ incubator for 2 hrs.
2. Two hours after plating, early adhering cells (mainly fibroblasts) will attach to the surface of the flask; label this cell population as pre-plate 1 (pp1).
3. Transfer the media containing the nonadherent cells into a second collagen type I-coated T-25 flask, label pp2. Return flasks to the incubator.
4. After 18 hrs, transfer the media from pp2 into a sterile 15 mL conical tube and centrifuge at $930 \times g$ at 4°C for 5 min. Aspirate out the supernatant and resuspend the cell pellet in 5 mL of PM and transfer the media containing the nonadherent cells into a third T-25 flask labeled pp3. Add 5 mL fresh PM to the pp2 flask. Return the flasks to the incubator.
5. Repeat Step 4 three times to obtain pp6. This last cell suspension can be maintained for up to 72 hrs to maximize adherence of the slowest adhering cells. Change the media on pp2–5 cells every other day and freeze as soon as a sufficient number is reached. Most of the cells in pp6 die, but the surviving cells will slowly begin to proliferate, creating small adherent colonies. These viable cells are small, round and refractive. The surviving cells at pp6 are termed muscle derived stem/progenitor cells (MDSPCs, *See Note 8*).

3.4 Expanding MDSPCs (Figure 2)

1. Remove the culture medium (PM) and wash the cells with HBSS.
2. Apply pre-warmed 0.25% Trypsin-EDTA in HBSS and gently tap the corners or bottom of the flasks stimulate cell detaching. After ~1 min, check the flask under the microscope to ensure that all of the cells are detached.
3. Inhibit the Trypsin-EDTA by adding PM (~3 mL for T-25 flasks). Transfer the cell suspension to a 15 mL tube and centrifuge at $930 \times g$ for 5 min at 4°C.
4. Aspirate off the supernatant and resuspend the pellet in PM. Count the cells and plate at a density of 500–1000 cells/cm² (e.g. ~12,000–25,000 cell in T-25 flask, *See Note 9*) in a fresh collagen type I-coated flask. Incubate the cells at 37 °C in a humidified, 5% CO₂ incubator. Maintain the cells at a low cell density (30% confluence), to minimize cell myogenic differentiation. MDSPCs need to be

⁸If contamination is observed, discard all cultures and clean the hood and incubators thoroughly with topical application of disinfectant (Coflikt, Decon Laboratories #4101). Autoclave all removable parts of the hood and incubator before starting a new isolation. It is not recommended to increase the dose of antibiotics or to use antimycotics since this may affect the stemness properties of MDSPCs.

trypsinized when small colonies are visible regardless of the overall cell density. MDSPCs usually remain quiescent after isolation; therefore, 3–4 weeks are needed to obtain sufficient cells for cryopreservation or experimentation (See Note 10).

3.5 Re-plating MDSPCs (Figure 2)

If pp6 cells contain a high percentage of large, flat, fibroblast-like cells, it is necessary to re-plate the cells as described below.

1. Follow steps 1–3 in part 3.4.
2. Aspirate out the supernatant and resuspend the pellet in PM, then plate the cells in a fresh collagen type I-coated T-25 flask. Allow the cells to adhere to the flask for 20–30 min in the incubator.
3. Collect the media containing the non-adherent cells and ‘re-plate’ them onto a new collagen type I-coated T-25 flask. Incubate the cells at 37°C in a humidified, 5% CO₂ incubator. Re-plating steps can be repeated several times if necessary.

3.6 Freezing MDSPCs

Freeze each preplate population (pp1–pp5) when the cells are 80% confluency. The pp6 can be frozen once a sufficient number of cells has been reached, while keeping the cells at low confluence (<30%) at all times. Cell pellets containing $\sim 1 \times 10^5$ – 3×10^5 cells should be mixed with freezing media (1:10 dilution of DMSO: FBS) and placed in –20°C for 2 hrs. The vials are then placed in the middle of two 15 mL Styrofoam boxes or a Mr. Frosty freezing container then transferred to a –80°C freezer. For long term preservation, the cells should be transferred to liquid nitrogen after 3 months.

3.7 Thawing MDSPCs

1. Transfer frozen vials of cells from the –80°C freezer or liquid nitrogen to the laboratory on dry ice.
2. Thaw the content of the vials using a 37°C water bath. Thawing needs to be done quickly (<1 min) because DMSO is toxic to the cells.
3. Transfer the content of the vial into a 15 mL conical tube containing 5 mL of PM and centrifuge at $930 \times g$ for 5 min at 4°C. Aspirate off the supernatant. Resuspend the cell pellet in a small volume of PM media. The contents of one cryovial ($\sim 1 \times 10^5$ /T-75) should be plated according to step 4 in section 3.4.
4. The day after thawing, change the media to remove the dead cells.

3.8 Measuring the quality/function of isolated MDSPCs (Table 1)

After isolation, the quality of MDSPC is assessed by measuring stem-like properties including expression of stem/progenitor markers profile, self-renewal, and multi-lineage differentiation (summarized in Table 1).

⁹The seeding density is critical and varies depending on the cell proliferation kinetics. Low cell density impedes cell proliferation and high density causes the cells to fuse (differentiate). The optimal density must be determined empirically (Figure 2). Cell proliferation rates vary depending on the sex, age and health of the donor as well as the amount muscle tissue available for cell isolation.

Consistency in cell isolation and culturing of MDSPCs is the key to maintaining pluripotency.

¹⁰Since MDSPCs are quiescent cells, it takes a minimum of 1–2 weeks for cells to show any sign of proliferation. This time varies depending upon the number of cells isolated at pp6. Trypsinization should be avoided until visible colonies are evident.

Acknowledgments

This work was supported by multiple institutes throughout many years including the National Institutes of Health, the US Department of Defense, the Muscular Dystrophy Association, the University of Pittsburgh Cancer Institute, and generous support from the William F. and Jean W. Donaldson Endowed Chair at the Children's Hospital of Pittsburgh of UPMC, and the Henry J. Mankin Endowed Chair for Orthopaedic Research at the University of Pittsburgh.

Reference

1. Pera MF. Scientific considerations relating to the ethics of the use of human embryonic stem cells in research and medicine. *Reprod Fertil Dev.* 2001; 13(1):23–29. [PubMed: 11545161]
2. Hook CC. In vitro fertilization and stem cell harvesting from human embryos: the law and practice in the United States. *Pol Arch Med Wewn.* 2010; 120(7–8):282–289. [PubMed: 20693959]
3. Blendon RJ, et al. The public, political parties, and stem-cell research. *N Engl J Med.* 2011; 365(20):1853–1856. [PubMed: 22087677]
4. Usas A, et al. Skeletal muscle-derived stem cells: implications for cell-mediated therapies. *Medicina (Kaunas).* 2011; 47(9):469–479. [PubMed: 22156603]
5. Mizuno H. Adipose-derived stem and stromal cells for cell-based therapy: current status of preclinical studies and clinical trials. *Curr Opin Mol Ther.* 2010; 12(4):442–449. [PubMed: 20677095]
6. Wilson A, et al. Adipose-derived stem cells for clinical applications: a review. *Cell Prolif.* 2011; 44(1):86–98. [PubMed: 21199013]
7. Carr LK, et al. 1-year follow-up of autologous muscle-derived stem cell injection pilot study to treat stress urinary incontinence. *Int Urogynecol J Pelvic Floor Dysfunct.* 2008; 19(6):881–883. [PubMed: 18204978]
8. Owen M. Marrow stromal stem cells. *J Cell Sci Suppl.* 1988; 10:63–76. [PubMed: 3077943]
9. da Silva Meirelles L, et al. Mesenchymal stem cells reside in virtually all postnatal organs and tissues. *J Cell Sci.* 2006; 119(Pt 11):2204–2213. [PubMed: 16684817]
10. Watts C, et al. Anatomical perspectives on adult neural stem cells. *J Anat.* 2005; 207(3):197–208. [PubMed: 16185244]
11. Lim DA, et al. The adult neural stem cell niche: lessons for future neural cell replacement strategies. *Neurosurg Clin N Am.* 2007; 18(1):81–82. ix. [PubMed: 17244556]
12. Baroffio A, et al. Identification of self-renewing myoblasts in the progeny of single human muscle satellite cells. *Differentiation.* 1996; 60(1):47–57. [PubMed: 8935928]
13. Qu-Petersen Z, et al. Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. *J Cell Biol.* 2002; 157(5):851–864. [PubMed: 12021255]
14. Sherwood RI, et al. Isolation of adult mouse myogenic progenitors: functional heterogeneity of cells within and engrafting skeletal muscle. *Cell.* 2004; 119(4):543–554. [PubMed: 15537543]
15. Shen W, et al. Adipose tissue quantification by imaging methods: a proposed classification. *Obes Res.* 2003; 11(1):5–16. [PubMed: 12529479]
16. Toma JG, et al. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol.* 2001; 3(9):778–784. [PubMed: 11533656]
17. Burke ZD, et al. Stem cells in the adult pancreas and liver. *Biochem J.* 2007; 404(2):169–178. [PubMed: 17488235]
18. Barker N, et al. Identification of stem cells in small intestine and colon by marker gene *Lgr5*. *Nature.* 2007; 449(7165):1003–1007. [PubMed: 17934449]
19. Yen TH, Wright NA. The gastrointestinal tract stem cell niche. *Stem Cell Rev.* 2006; 2(3):203–212. [PubMed: 17625256]
20. Deasy BM, et al. Long-term self-renewal of postnatal muscle-derived stem cells. *Mol Biol Cell.* 2005; 16(7):3323–3333. [PubMed: 15872085]
21. Lavasani M, et al. Muscle-derived stem/progenitor cell dysfunction limits healthspan and lifespan in a murine progeria model. *Nat Commun.* 2012; 3:608. [PubMed: 22215083]

22. Yokoyama T, et al. Autologous primary muscle-derived cells transfer into the lower urinary tract. *Tissue Eng.* 2001; 7(4):395–404. [PubMed: 11506729]
23. Chancellor MB, et al. Gene therapy strategies for urological dysfunction. *Trends Mol Med.* 2001; 7(7):301–306. [PubMed: 11425638]
24. Musgrave DS, et al. Human skeletal muscle cells in ex vivo gene therapy to deliver bone morphogenetic protein-2. *J Bone Joint Surg Br.* 2002; 84(1):120–127. [PubMed: 11837817]
25. Sakai T, et al. The use of ex vivo gene transfer based on muscle-derived stem cells for cardiovascular medicine. *Trends Cardiovasc Med.* 2002; 12(3):115–120. [PubMed: 12007736]
26. Peng H, et al. Synergistic enhancement of bone formation and healing by stem cell-expressed VEGF and bone morphogenetic protein-4. *J Clin Invest.* 2002; 110(6):751–759. [PubMed: 12235106]
27. Ikezawa M, et al. Dystrophin delivery in dystrophin-deficient DMDmdx skeletal muscle by isogenic muscle-derived stem cell transplantation. *Hum Gene Ther.* 2003; 14(16):1535–1546. [PubMed: 14577915]
28. Hannallah D, et al. Retroviral delivery of Noggin inhibits the formation of heterotopic ossification induced by BMP-4, demineralized bone matrix, and trauma in an animal model. *J Bone Joint Surg Am.* 2004; 86-A(1):80–91. [PubMed: 14711949]
29. Deasy BM, et al. Effect of VEGF on the regenerative capacity of muscle stem cells in dystrophic skeletal muscle. *Mol Ther.* 2009; 17(10):1788–1798. [PubMed: 19603004]
30. Peng H, et al. VEGF improves, whereas sFlt1 inhibits, BMP2-induced bone formation and bone healing through modulation of angiogenesis. *J Bone Miner Res.* 2005; 20(11):2017–2027. [PubMed: 16234975]
31. Chirieleison SM, et al. Human muscle-derived cell populations isolated by differential adhesion rates: phenotype and contribution to skeletal muscle regeneration in Mdx/SCID mice. *Tissue Eng Part A.* 2012; 18(3–4):232–241. [PubMed: 21854253]
32. Okada M, et al. Human skeletal muscle cells with a slow adhesion rate after isolation and an enhanced stress resistance improve function of ischemic hearts. *Mol Ther.* 2012; 20(1):138–145. [PubMed: 22068427]
33. Torrente Y, et al. Intraarterial injection of muscle-derived CD34(+)Sca-1(+) stem cells restores dystrophin in mdx mice. *J Cell Biol.* 2001; 152(2):335–348. [PubMed: 11266450]
34. Lee JY, et al. Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. *The Journal of cell biology.* 2000; 150(5):1085–1100. [PubMed: 10973997]
35. Wright V, et al. BMP4-expressing muscle-derived stem cells differentiate into osteogenic lineage and improve bone healing in immunocompetent mice. *Mol Ther.* 2002; 6(2):169–178. [PubMed: 12161183]
36. Kuroda R, et al. Cartilage repair using bone morphogenetic protein 4 and muscle-derived stem cells. *Arthritis Rheum.* 2006; 54(2):433–442. [PubMed: 16447218]
37. Aguiari P, et al. High glucose induces adipogenic differentiation of muscle-derived stem cells. *Proc Natl Acad Sci U S A.* 2008; 105(4):1226–1231. [PubMed: 18212116]
38. Arriero M, et al. Adult skeletal muscle stem cells differentiate into endothelial lineage and ameliorate renal dysfunction after acute ischemia. *Am J Physiol Renal Physiol.* 2004; 287(4):F621–F627. [PubMed: 15198930]
39. Jackson KA, et al. Hematopoietic potential of stem cells isolated from murine skeletal muscle. *Proc Natl Acad Sci U S A.* 1999; 96(25):14482–14486. [PubMed: 10588731]
40. Romero-Ramos M, et al. Neuronal differentiation of stem cells isolated from adult muscle. *J Neurosci Res.* 2002; 69(6):894–907. [PubMed: 12205682]
41. Vourc'h P, et al. Isolation and characterization of cells with neurogenic potential from adult skeletal muscle. *Biochem Biophys Res Commun.* 2004; 317(3):893–901. [PubMed: 15081424]
42. Winitsky SO, et al. Adult murine skeletal muscle contains cells that can differentiate into beating cardiomyocytes in vitro. *PLoS Biol.* 2005; 3(4):e87. [PubMed: 15757365]
43. Arsic N, et al. Muscle-derived stem cells isolated as non-adherent population give rise to cardiac, skeletal muscle and neural lineages. *Exp Cell Res.* 2008; 314(6):1266–1280. [PubMed: 18282570]

44. Tamaki T, et al. Identification of myogenic-endothelial progenitor cells in the interstitial spaces of skeletal muscle. *J Cell Biol.* 2002; 157(4):571–577. [PubMed: 11994315]
45. Cao B, et al. Muscle stem cells differentiate into haematopoietic lineages but retain myogenic potential. *Nat Cell Biol.* 2003; 5(7):640–646. [PubMed: 12792651]
46. Lee JY, et al. Effect of bone morphogenetic protein-2-expressing muscle-derived cells on healing of critical-sized bone defects in mice. *J Bone Joint Surg Am.* 2001; 83-A(7):1032–1039. [PubMed: 11451972]
47. Matsumoto T, et al. Cartilage repair in a rat model of osteoarthritis through intraarticular transplantation of muscle-derived stem cells expressing bone morphogenetic protein 4 and soluble Flt-1. *Arthritis Rheum.* 2009; 60(5):1390–1405. [PubMed: 19404941]
48. Oshima H, et al. Differential myocardial infarct repair with muscle stem cells compared to myoblasts. *Mol Ther.* 2005; 12(6):1130–1141. [PubMed: 16125468]
49. Payne TR, et al. Regeneration of dystrophin-expressing myocytes in the mdx heart by skeletal muscle stem cells. *Gene Ther.* 2005; 12(16):1264–1274. [PubMed: 15843810]
50. Lee JY, et al. The effects of periurethral muscle-derived stem cell injection on leak point pressure in a rat model of stress urinary incontinence. *Int Urogynecol J Pelvic Floor Dysfunct.* 2003; 14(1): 31–37. discussion 37. [PubMed: 12601514]
51. Cannon TW, et al. Improved sphincter contractility after allogenic muscle-derived progenitor cell injection into the denervated rat urethra. *Urology.* 2003; 62(5):958–963. [PubMed: 14624934]
52. Chermansky CJ, et al. Intraurethral muscle-derived cell injections increase leak point pressure in a rat model of intrinsic sphincter deficiency. *Urology.* 2004; 63(4):780–785. [PubMed: 15072911]
53. Kwon D, et al. Periurethral cellular injection: comparison of muscle-derived progenitor cells and fibroblasts with regard to efficacy and tissue contractility in an animal model of stress urinary incontinence. *Urology.* 2006; 68(2):449–454. [PubMed: 16904482]
54. Gussoni E, et al. Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature.* 1999; 401(6751):390–394. [PubMed: 10517639]
55. Jankowski RJ, et al. Flow cytometric characterization of myogenic cell populations obtained via the preplate technique: potential for rapid isolation of muscle-derived stem cells. *Hum Gene Ther.* 2001; 12(6):619–628. [PubMed: 11426462]
56. Sherley JL, et al. A quantitative method for the analysis of mammalian cell proliferation in culture in terms of dividing and non-dividing cells. *Cell Prolif.* 1995; 28(3):137–144. [PubMed: 7734623]
57. Deasy BM, et al. Modeling stem cell population growth: incorporating terms for proliferative heterogeneity. *Stem Cells.* 2003; 21(5):536–545. [PubMed: 12968108]
58. Hoffman EP, et al. Conservation of the Duchenne muscular dystrophy gene in mice and humans. *Science.* 1987; 238(4825):347–350. [PubMed: 3659917]
59. d'Albis A, et al. Regeneration after cardiotoxin injury of innervated and denervated slow and fast muscles of mammals. Myosin isoform analysis. *Eur J Biochem.* 1988; 174(1):103–110. [PubMed: 3371354]
60. Matsuura T, et al. Skeletal muscle fiber type conversion during the repair of mouse soleus: potential implications for muscle healing after injury. *J Orthop Res.* 2007; 25(11):1534–1540. [PubMed: 17593537]
61. Lavasani M, et al. Nerve growth factor improves the muscle regeneration capacity of muscle stem cells in dystrophic muscle. *Hum Gene Ther.* 2006; 17(2):180–192. [PubMed: 16454652]

Figure 1.

Isolation of muscle-derived stem/progenitor cells (MDSPCs). Skeletal muscle is mechanically and enzymatically dissociated to a single cell suspension. The muscle cells are resuspended in proliferation medium and plated on collagen type I-coated flasks. Different populations of muscle-derived cells are isolated based on their adhesion characteristics. Pre-plate 1 (pp1) cells adhere in the first 2 hrs after isolation and consist predominantly of primary fibroblasts. Subsequent pre-plates obtained at 18–24 hrs intervals (pp2–5), contain a mixture of fibroblast and myoblast-like cells. Cells in pp6 take an additional 72 hrs to attach. Most of the cells from pp6 die during the first 1–2 wks of culturing. The few adherent cells that survive proliferate to form colonies. These viable, proliferating pp6 cells are called MDSPCs.

Figure 2. MDSPC morphology and maintenance. Representative images indicating (a) the initial density of MDSPCs yielded at pp6 (~200 cells/cm²). (b) MDSPCs maintained at an optimal confluence of <30 %. (c) MDSPCs in need of trypsinization and splitting 1:2. (d) MDSPCs ready to be re-plated to eliminate the fibroblast-like cells (arrows). Images are at 5X magnification.

Endpoints used to evaluate the quality and function of MDSPCs and how those parameters change in cells isolated from aged organisms

MDSPCs are double positive for Sc α 1 and CD34 and negative for CD45 (13, 21, 34). Although, variability and alteration in the expression levels has been observed as a consequence of *in vitro* culturing (55). Growth kinetics (proliferation and/or self-renewal) is measured using a live-cell imaging (LCI) system. This system allows for time-lapsed imaging of single cells or colonies over long periods of time, so that growth and differentiation kinetics can be accurately measured (20, 56, 57). To evaluate the differentiation potential of MDSPCs, the cells are switched to specific differentiation media for myogenic, osteogenic, chondrogenic, and adipogenic lineages. Subsequently the cultures are analyzed for lineage-specific morphology and differentiation markers (21). The function (regenerative capacity) of MDSPCs is evaluated *in vivo* by injecting the cells into the gastrocnemius muscle of *mdx*/SCID mice, a mouse model of Duchenne muscular dystrophy with profound muscle degeneration due to lack of dystrophin expression (58) that are also immunocompromised (SCID). Two weeks after injecting MDSPCs, the muscle is harvested and stained for dystrophin to detect donor-derived myofibers. Alternatively, muscle regeneration can be tested in wild-type mice following cardiotoxin injury (21, 59). In this case, regenerating fibers are identified by centronucleation (21, 60).

Table 1

	Assay used	Unit	Ref.	Young murine WT-MDSPCs	Aged murine MDSPCs
Marker profile Sc α 1+ CD34+ CD45-	Immuno: RT-PCR	% cells of total population	(21)	18% of cells at pp3 (host 3 wks old); 9% at pp3 (host 4–5 mths old)	3% of cells at pp3 (host 3 yrs old)
Proliferation	Live-cell imaging (LCI) system	DT (hrs) PDT (hrs)	(21)	DT = 13 $\pm$ 2 hrs PDT = 15 hrs (host 3 wks old)	DT = 16 $\pm$ 6 hrs PDT = 18 hrs (host 2 yrs old)
Differentiation	myogenic osteogenic chondrogenic adipogenic	+/- differentiation capacity	(13,21)	+ for all four lineages	adipocyte > myocytes > chondrocyte > osteocyte
Engraftment potential and regeneration	IM injection into <i>mdx</i> /SCID mice or WT mice after cardiotoxin injury	# or % of regenerating myofibers and their cross- sectional area	(13,21,61)	70% of regenerating, centronucleated fibers have an area >1,000 μ m 2	70% of regenerating, centronucleated fibers have an area <750 μ m 2

DT: division time; FACS: Flow cytometry; IM: intramuscular injection; Immuno: immunocytochemistry; LCI: live cell imaging; PDT: population-doubling time; FMyHC: fast myosin heavy chain; WT: wild-type