

Isolation and Characterization of Vessel-Associated Stem/Progenitor Cells from Skeletal Muscle

Rossana Tonlorenzi, Giuliana Rossi, and Graziella Messina

Abstract

More than 10 years ago, we isolated from mouse embryonic dorsal aorta a population of vessel-associated stem/progenitor cells, originally named mesoangioblasts (MABs), capable to differentiate in all mesodermal-derived tissues, including skeletal muscle. Similar though not identical cells have been later isolated and characterized from small vessels of adult mouse and human skeletal muscles. When delivered through the arterial circulation, MABs cross the blood vessel wall and participate in skeletal muscle regeneration, leading to an amelioration of muscular dystrophies in different preclinical animal models. As such, human MABs have been used under clinical-grade conditions for a Phase I/II clinical trial for Duchenne muscular dystrophy, just concluded. Although some pericyte markers can be used to identify mouse and human MABs, no single unequivocal marker can be used to isolate MABs. As a result, MABs are mainly defined by their isolation method and functional properties. This chapter provides detailed methods for isolation, culture, and characterization of MABs in light of the recent identification of a new marker, PW1/Peg3, to screen and identify competent MABs before their use in cell therapy.

Key words Mesoangioblasts, Muscle stem cells, Pericytes, Mesodermal lineages, Cell culture, PW1/Peg3

1 Introduction

Skeletal muscle regeneration is assured by satellite cells (SCs), which are localized between the basal lamina and the sarcolemmal membrane [1]. Despite their indispensable role in the regeneration of adult skeletal muscle [2–6], in the last years, several groups identified different atypical muscle progenitors of non-somitic origin that are able to differentiate in skeletal muscle [7–18]. Among these, in the last years mesoangioblasts (MABs) demonstrated to be the most promising. MABs are blood vessel-associated stem/progenitor cells that can differentiate into mesoderm cell types, including skeletal muscle [7]. Most importantly, when systemically delivered, MABs cross the blood vessel wall and participate in skeletal muscle regeneration, leading to an amelioration of muscular dystrophies in different preclinical models [19–23]. The ability of MABs to cross the

vessel wall confers an advantage as therapeutic donor stem cells as compared with SCs and myoblasts that need to be delivered directly into the muscle tissue to properly engraft [24, 25]. Consistently, human MABs, expanded under clinical-grade conditions, have been recently used for a Phase I/II clinical trial for Duchenne muscular dystrophy (EudraCT no. 2011-000176-33; [26]).

Both human and mouse MABs can be retrospectively characterized by a combination of markers not all shared by both cell populations (Table 1).

This chapter provides an updated guide for isolation, expansion, and characterization of MABs from adult human and mouse skeletal muscle (*see* Subheadings 3.1–3.3 and 3.9). Various differentiation methods are also described: spontaneous skeletal muscle differentiation (*see* Subheadings 3.5), induction of smooth muscle

Table 1
List of the positive and negative markers used to characterize human and mouse MABs

Marker	mMABs	hMABs
Sca-1	+	NA
c-kit	–	–
CD45 (leukocyte common antigen)	–	–
CD34	–	–
CD31 (PECAM-1)	–	–
VE-cadherin	–	–
Tie2	–	–
Pax3	+	+
Pax7	–	–
Myf5	–	–
MyoD	–	–
PW1/Peg3	+	+
Alkaline phosphatase	+/-	+/-
Ng2	+	+
Pdgfrb	+	+
CD44 (hyaluronan-binding protein)	+	+
CD56 (NCAM)	–	–
CD133 (prominin-1)	–	–
CD146 (MCAM)	+/-	+/-

by TGF- β (*see* Subheading 3.6), induction of osteoblasts by BMP2 (*see* Subheading 3.7), and induction of adipocytes (*see* Subheading 3.8). Additionally, in light of the recent identification of PW1/Peg3 as a key marker essential in regulating MAB competence [27], a paragraph will be dedicated to the description of the methods developed to detect PW1/Peg3 in mouse and human MABs (*see* Subheading 3.9).

Successful isolation, propagation, and characterization of MABs require basic animal handling and competences in cellular and molecular biology. Notably, for human MABs, cell cultures must be cultured under physiological O₂ conditions (3 % O₂, 5 % CO₂, 92 % N₂). Importantly, all the procedure described in this chapter should be performed under sterile conditions in either Class II biohazard flow hoods (especially for human cells) or laminar flow horizontal hoods. For mouse samples, the Institutional Animal Welfare Body (AWB), Ethics Committee, and National Local Authority must approve the protocols. Muscle biopsies must be performed under general or local anesthesia with the minimum degree of pain. Approval of Institutional Ethics Committee and patients' informed consent are necessary in case of human samples.

2 Materials

2.1 Basic Materials, Media, and Solutions

1. 5 % CO₂, 3 % O₂, 92 % N₂ humidified (water-saturated) incubator.
2. Biohazard flow hood.
3. Benchtop centrifuge.
4. Inverted phase-contrast microscope.
5. Stereoscopic microscope (optional).
6. Water bath.
7. +4 °C refrigerator.
8. -20° freezer.
9. -80° freezer.
10. Liquid nitrogen tank.
11. *Collagen solution (250 ml) and Petri dish coating*: transfer 250 mg of lyophilized collagen type 1 to a sterile glass bottle. Gradually add 50 ml of glacial acetic acid. Due to variable purity in different collagen preparations, the time necessary for complete dissolution of collagen may vary. Overnight incubation at room temperature is recommended. After complete collagen dissolution in acetic acid, gradually add 200 ml of ultrapure distilled water. Mix gently without shaking. Store up to 6 months at 4 °C.

To obtain an efficient solution, it is very important to wait for the collagen to be completely dissolved in acetic acid before adding ultrapure distilled water.

Membrane filtration is not recommended (a substantial portion of collagen will be lost or degraded).

For coating, place the appropriate number of Petri dishes under a biohazard flow hood. Carefully add the collagen type I solution into each Petri dish making sure the whole surface is completely covered. Use 1, 5, and 10 ml of collagen solution for 3.5-, 6-, and 9-cm petri dish, respectively, and let stand 5 min. Slowly remove most of the solution (80–90 %), leaving the surface of the dish uniformly wet and let dry completely.

12. *Collagenase/Dispase solution (50 ml)*: depending on enzyme activity U/W (specified by the manufacturer for each lot), weigh the appropriate amounts to prepare 50 ml of 1 U/ml collagenase, 0.5 U/ml Dispase II stock solution in PBS. Filter through a 0.22- μ m syringe filter and store in 5-ml aliquots up to 6 months at -20°C .
13. *D2 medium (250 ml)*: 245 ml high-glucose DMEM supplemented with 5 ml heat-inactivated horse serum (HS, Euroclone), 2 mM glutamine, 1 % penicillin/streptomycin solution (10,000 U/ml and 10 μ g/ml, respectively), 1 mM sodium pyruvate.
Store up to 4 weeks at $+4^{\circ}\text{C}$.
14. *D20 medium (250 ml)*: 200 ml high-glucose DMEM supplemented with 50 ml heat-inactivated fetal bovine serum (FBS, Euroclone), 2 mM glutamine, 1 % penicillin/streptomycin solution (10,000 U/ml and 10 μ g/ml, respectively), and 1 mM sodium pyruvate.
Store up to 4 weeks at $+4^{\circ}\text{C}$.
15. *M5 medium (250 ml)*: 237.5 ml Megacell DMEM supplemented with 12.5 ml heat-inactivated FBS, 2 mM glutamine, 1 % penicillin/streptomycin solution (10,000 U/ml and 10 μ g/ml, respectively), 0.1 mM β -mercaptoethanol, 1 % non-essential amino acids, and 1.25 μ g human recombinant bFGF (Life Technologies).
Store up to 2 weeks at $+4^{\circ}\text{C}$.
16. *Matrigel stock and Petri dish coating*.
Thaw a 10-ml bottle of growth factor-reduced Matrigel (Corning) overnight on ice. Prepare aliquots by using sterile microcentrifuge tubes chilled on ice and pipet tips kept at $+4^{\circ}\text{C}$. Store undiluted Matrigel in 100- μ l aliquots up to 12 months at -20°C .
Concentrated Matrigel solution tends to polymerize very quickly at room temperature.

Frozen aliquots have to be thawed on ice and immediately diluted in cold sterile solutions.

Gentle and slow pipetting is recommendable for either diluted or concentrated Matrigel solutions, to avoid degradation of the components. Matrigel solutions cannot be membrane filtered.

For Petri coating, thaw Matrigel stock solution on ice and prepare the working solution by diluting the stock 1:80 in cold DMEM (without any supplement). Place the appropriate number of Petri dishes to be coated under a hood and apply Matrigel working solution carefully into each Petri dish making sure the whole surface is completely covered.

Use 1, 3, and 7 ml of Matrigel working solution for 3.5-, 5-, and 9-cm Petri dishes, respectively, and incubate 30 min–2 h at 37 °C. Just before use, remove the Matrigel working solution and gently rinse the dish surface with appropriate culture medium. Matrigel-coated Petri dishes have to be freshly prepared.

Diluted Matrigel working solution can be stored up to 24 h at +4 °C.

17. Human recombinant bFGF stock solution (1 ml): reconstitute 50 µg of human recombinant bFGF (Life Technologies) in 1 ml of 10 mM Tris–HCl, pH 7.6.

Store in 25-µl aliquots up to 6 months at –20 °C.

18. *Freezing solution (50 ml)*: 45 ml heat-inactivated FBS supplemented with 5 ml Hybri-Max DMSO. Prepare fresh.

2.2 Isolation, Propagation, and Freezing of MABs from Human Skeletal Muscle (hMABs)

1. Human skeletal muscle fragments (at least 200 mg of tissue; *see Note 1*).
2. Ca²⁺/Mg²⁺ free phosphate-buffered saline (CMF-PBS).
3. M5 medium.
4. 6- and 15-cm Petri dishes (Nunc).
5. 3.5- and 6-cm collagen-coated Petri dishes.
6. Rounded-edge disposable scalpels.
7. Curved forceps.
8. Sharp-edged straight forceps.
9. 0.05 % (w/v) trypsin/0.02 % (w/v) EDTA.
10. 25, 75-cm² tissue culture flasks (vented, Nunc).
11. M5 medium.
12. Freezing solution.
13. Hemacytometer.
14. 1.8-ml sterile cryovials , ice cold.
15. Cryogenic-controlled rate freezing container or insulated cardboard/polystyrene foam box.

**2.3 CD56+ Cell
Fraction Depletion by
Magnetic Microbeads
Separation**

1. Trypsin/EDTA.
2. Ca²⁺/Mg²⁺ free phosphate-buffered saline (CMF-PBS).
3. M5 medium.
4. CD56 microbeads, human (Miltenyi).
5. LD columns (Miltenyi).
6. MidiMACS MultiStand (Miltenyi).
7. MidiMACS Separator (Miltenyi).
8. 40- μ m cell strainers.
9. Trypan blue.
10. Hemocytometer.
11. *Buffer for microbeads separation (500 ml)*: CMF-PBS (pH 7.2) supplemented with 0.5 % BSA and 2 mM EDTA. Store up to 4 weeks at +4 °C.

**2.4 Isolation,
Propagation,
and Freezing of MABs
from Murine Skeletal
Muscle (mMABs)**

1. Mouse skeletal (tibialis anterior) muscle fragments (at least 50 mg of tissue; *see Note 1*).
2. Ca²⁺/Mg²⁺ free phosphate-buffered saline (CMF-PBS).
3. D20 medium.
4. 6- and 15-cm Petri dishes (Nunc).
5. 3.5- and 6-cm collagen-coated Petri dishes.
6. Matrigel-coated 48-well multiwells.
7. Rounded-edge disposable scalpels.
8. Curved forceps.
9. Sharp-edged straight forceps.
10. 0.05 % (w/v) trypsin/0.02 % (w/v) EDTA.
11. 25, 75-cm² tissue culture flasks (vented, Nunc).

2.5 AP Staining

1. Human or murine MAB cultures (grown in 3.5-cm Petri dishes).
2. CMF-PBS.
3. *AP buffered solution, pH 9.5 (10 ml)*: 10 ml of 100 mM Tris-HCl (pH 9.5), additioned with 100 mM NaCl, 50 mM MgCl₂, 0.1 % (v/v) Tween 20. Adjust pH with 0.1 N HCl or 0.1 N NaOH. Particular attention must be paid to the exact pH (9.5). Prepare fresh.
4. Alkaline phosphatase (AP) staining solution (10 ml): dissolve 135 μ g of 4-nitroblue tetrazolium chloride (NTC, Roche) and 1.75 mg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Roche) in AP buffered solution. Prepare fresh.

2.6 Skeletal Muscle Differentiation

1. MABs to be tested grown in a 25-cm² flask.
2. M5/D20 medium.
3. 3.5-cm Petri dishes, coated with growth factor-reduced Matrigel.
4. 3.5-cm elastic surface (ESS) Petri dishes (IBIDI), coated with growth factor-reduced Matrigel.
5. CMF-PBS.
6. D2 medium.

2.7 Induction into Smooth Muscle by TGF- β 1 Treatment

1. MABs to be tested grown in a 25-cm² flask.
2. M5 medium/D20 medium.
3. D2 medium.
4. *Human recombinant TGF- β stock solution (1 ml)*: reconstitute 5 μ g human recombinant TGF- β (Life Technologies) in 1 ml of 10 mM citric acid, additioned with 0.1 % BSA.
Store in 25- μ l aliquots up to 6 months at -20 °C.

2.8 Induction of Osteoblast Differentiation by BMP2 Treatment

1. MABs to be tested grown in a 25-cm² flask.
2. M5 medium/D20 medium.
3. D2 medium.
4. *Human recombinant BMP2 stock solution (1 ml)*: reconstitute 100 μ g human recombinant BMP2 (Life Technologies) in 1 ml of 20 mM Acetic Acid.
Store in 25- μ l aliquots up to 6 months at -20 °C.

2.9 Induction of Adipocyte Differentiation

1. MABs to be tested grown in a 25-cm² flask.
2. M5 medium/D20 medium.
3. Adipogenic medium (LONZA cat. no. 3004).
4. *Oil Red O solution*: dissolve 350 mg of Oil Red O powder in 100 ml of 2-propanol in a glass bottle. Let stand overnight at room temperature, protected from light. Do not mix. Filter on 3-MM chromatography paper into a new glass bottle. Add 75 ml of distilled water. Let stand overnight at +4 °C, protected from light. Do not mix. Filter two times through 3-MM chromatography paper into a new glass bottle.
Store up to 6 months at room temperature, protected from light. Oil Red O is a very strong staining agent and should be handled carefully according to the manufacturer's product data sheet instructions.

**2.10 PW1/Peg3
as a Screening Molecule
to Identify the Best
Mesoangioblast
Populations**

**2.10.1 Real-Time PCR
for Murine and Human
PW1/ Peg3**

For general information, refer to [27, 28].

Maintain all components on ice while preparing PCR mix; store solutions containing Sybr Green protected from light. Clean bench and pipettes before starting with the procedure and eventually use DNAZap for cleaning.

1. cDNA retrotranscribed from 1 µg of RNA, using random primers (iScript reverse transcription supermix, Biorad). cDNA should be diluted in nuclease-free water 1:10 before use.
2. Nuclease-free water.
3. PW1/Peg3-specific primers:
 Mouse Forward: GAGAATCCTCCATTTATATC
 Mouse Reverse: TCATGAATCTTCTGGTGCTC
 Human Forward: GATCCAAGAGAAGTGCCTACC
 Human Reverse: GGAAGATTCATCTTCACAAATCCC
 Reference gene-specific primers; we use GAPDH to normalize expression:
 Forward: TTCACCACCATGGAGAAGGC
 Reverse: GGCATGGACTGTGGTCATGA
4. Real-Time PCR Thermal Cycler (CFX-96 Connect, Biorad).
5. Optically clear PCR tubes or plates.
6. Sybr Green Master Mix (iTaq Universal SYBR Green Supermix (Biorad) for murine PW1 and Sso Advanced Universal SYBR Green Supermix (Biorad) for human Peg3).

**2.10.2 Immuno-
fluorescence for Murine
PW1**

1. mMABs Petri dish.
2. 4 % paraformaldehyde solution in PBS.
3. +4 °C refrigerator.
4. -20 °C freezer.
5. Precooled (at -20 °C) methanol.
6. *Blocking solution*: PBS additioned with 5 % goat serum, 2 % Bovine Serum Albumin (BSA).
7. *0.1 % BSA solution*: PBS additioned with 0.1 % BSA.
8. *5 % BSA solution*: PBS additioned with 5 % BSA.
9. Rabbit anti-mouse PW1 primary antibody (*see Note 2*).
10. Fluorophore-conjugated goat anti-rabbit secondary antibody.
11. DAPI solution.

12. Fluorescence mounting medium (Dako fluorescence mounting medium).
13. Fluorescence microscope.

2.10.3 Western Blot for Murine PW1

1. Murine MABs.
2. *RIPA lysis buffer*: PBS added with 1 % NP40, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 mM PMSF, 3.5 mM Na₃VO₄, proteases, and phosphatases inhibitors.
3. Ice.
4. Refrigerated centrifuge.
5. 1.5-ml tubes.
6. *2× loading buffer*: 100 mM Tris-HCl pH 6.8 added with 200 mM DTT, 4 % SDS, 0.2 % bromophenol blue, and 20 % glycerol (MilliQ water to volume).
7. 6 % acrylamide gel.
8. *Running buffer pH 8.3*: 125 mM Tris hydroxymethylamino-methane added with 1.25 M glycine and 0.5 % SDS (MilliQ water to volume).
9. Western blot electrophoretic apparatus.
10. iBlot Dry Blotting System (Life Technologies).
11. *5 % skimmed milk solution*: PBS added with 5 % skimmed milk and 0.05 % Tween 20.
12. Rabbit anti-mouse PW1 primary antibody (the same antibody used for the immunofluorescence).
13. HRP-conjugated goat anti-rabbit secondary antibody (Biorad).
14. *T-PBS*: PBS added with 0.05 % Tween 20.
15. ECL Western blot detection reagent.
16. X-ray developer, developing liquids, or digital chemiluminescence imaging system (ChemiDoc, Biorad).

3 Methods

The preliminary step for the isolation of both human and murine mesoangioblasts (hMABs and mMABs) is a short-time primary culture of skeletal muscle fragments.

Aim of this outgrowth phase is to increase, by selective culture conditions and procedures, the proportion of MAB populations originating from tissue explants.

Notably, the presence of CD56 expression reveals myogenic progenitors that may be derived from contaminating satellite cells or, alternatively, from in vitro differentiation of hMABs into satellite cells (lineage-tracing studies in *Tg:TN-AP-CreERT2* mouse

model indicate that this event may occur spontaneously during postnatal unperturbed development of skeletal muscle) [29]. CD56+ cell fraction depletion can be performed either by magnetic microbeads separation (as described in the Subheading 3.2) or by FACS sorting procedures. Notably, magnetic microbead separation is a fast purification step that does not affect cell viability, can be repeated more than once (to increase cell purity), and is adaptable to GMP protocols.

Efficiency in derivation of MABs may vary depending on age and intrinsic characteristics of skeletal muscle fragments. The accurate selection of tissue portions may however help to obtain optimal yield for each sample.

Sterile and tissue culture-grade reagents are recommended for all steps described.

All reagents are provided by Sigma, unless otherwise specified.

3.1 Isolation, Propagation, and Freezing of MABs from Human Skeletal Muscle (hMAB)

1. Rinse each skeletal muscle fragment in CMF-PBS to remove residual blood.
2. Set a 10-ml Petri dish containing 3–4 ml of M5 medium to start dissection.
3. Cut each fragment in 2 mm pieces using a round-edge scalpel, with the help of straight forceps (*see Note 3*).
4. Pretreat 6-cm diameter collagen-coated Petri dish by pipetting 2 ml of M5 medium into each dish, making sure that the surface is completely covered. Remove most of the medium, leaving the dish thoroughly wet.
5. Transfer the selected fragments into each dish (5–10 fragment/dish) with the help of curved forceps.
6. Add 2 ml of pre-warmed M5 medium pipetting it along the edge of the dish to prevent detachment and floating of fragments.
Incubate overnight in a 37 °C, 5 % CO₂, 3 % O₂, and 92 % N₂ humidified incubator (*see Notes 4 and 5*).
7. Around 24 h after initiation of cultures, carefully add two additional ml of pre-warmed M5 medium to each dish.
8. After 5–7 days, examine the cultures for preliminary growth of adherent cells.
9. Add 1.5 ml of pre-warmed M5 medium to each dish.
10. After additional 2–3 days, examine the cultures for hMABs: MABs initially look round and small, and then they tend to attach to the substrate as more flat/spindle-shaped cells. With passages, the floating fraction tends to reduce, and the adherent fraction increases in percentage (Fig. 1A).

11. Carefully transfer culture medium and floating cells to a new, uncoated 6-cm Petri dish.
In case of poor recovery of floating cells, transfer them to a smaller Petri dish (3.5 cm) to increase cell density and favor proliferation.
12. Add pre-warmed fresh medium if necessary (to reach a total volume of 5 ml).
13. After 24 h, examine the cultures. Around 50–70 % of the cells should adhere to the plastic surface, but a floating fraction should always be clearly distinguishable (*see* **Notes 6** and **7**).
14. When the adherent fraction of the cell population reaches 70–80 % confluence, proceed to trypsinization and transfer to 25-cm² flask.
15. At 70–80 % confluence of the adherent cell population, remove culture medium and set aside in 15-ml centrifuge tubes.
16. Rinse the growing surface with 2 ml CMF-PBS.
17. Add 2 ml of trypsin/EDTA and incubate 3–5 min at room temperature. Check under a microscope for complete detachment of cells.
18. Use the medium set aside to collect all cells. In this way, both floating and adherent populations are recovered.
19. Centrifuge 10 min at $188 \times g$, room temperature.
20. Gently suspend the pellet in 6-ml M5 medium and transfer to a single 75-cm² tissue culture flask or dispense 2-ml aliquots of cell suspension into each of the three 25-cm² flasks (1:3 split).
21. Add M5 medium to reach a final volume of 5 ml for 25-cm² tissue culture flasks (12 ml for 75-cm² tissue culture flasks) (*see* **Notes 8–10**).
22. To freeze hMABs, detach cells with trypsin/EDTA according to corresponding steps described for cell propagation (*see* **Note 11**).
23. Spin 5 min at $227 \times g$, discard supernatant and suspend the cell pellet in 5 ml of M5 medium.
24. Count cells with a hemocytometer.
25. Centrifuge 5 min at $227 \times g$, room temperature.
26. Discard supernatant and gently suspend cells in appropriate volume of cold freezing solution (optimal range of cell concentration: $1\text{--}2 \times 10^6$ cells/ml).
27. Set up the appropriate number of 1.8-ml cryovials and dispense 1 ml of cell suspension into each.
Each cryovial should be clearly labeled with date, cell line code, and passage number.
28. Transfer vials into a freezing container and place overnight at -80°C .

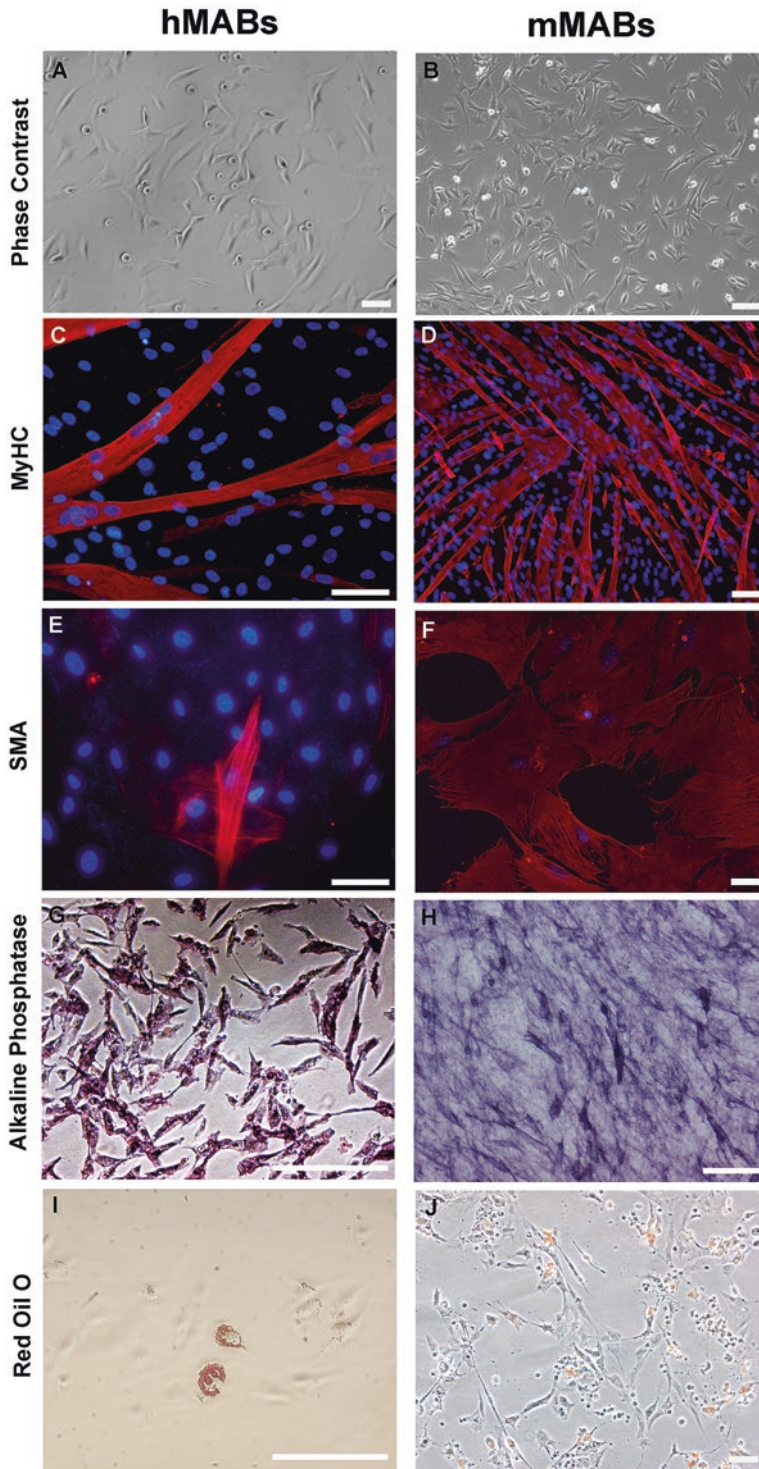


Fig. 1 Differentiation of mouse and human MABs into different mesodermal-derived cell types. (**A, B**) Phase-contrast images of growing human (**A**) and mouse (**B**) MABs; (**C, D**) immunofluorescence for all sarcomeric myosins (MyHC, in *red*) on differentiated human (**C**) and mouse (**D**) MABs; (**E, F**) smooth muscle actin (SMA, in *red*) immunofluorescence analysis of human (**E**) and mouse (**F**) MABs after TGF- β 1 stimulation; (**G, H**) alkaline phosphatase staining after BMP2 treatment of human (**G**) and mouse (**H**) MABs; (**I, J**) Oil Red O staining of human (**I**) and mouse (**J**) MABs after culturing in adipogenic medium. Scale bars 100 μ m

29. On the following day, transfer vials to $-135\text{ }^{\circ}\text{C}$ or to a liquid nitrogen container (*see Note 12*).

The morphology of hMABs after few passages in culture at the inverted phase contrast microscope is shown in Fig. 1A.

3.2 CD56+ Cell Fraction Depletion by Magnetic Microbead Separation

1. Trypsinize hMABs as described in Subheading 3.1.
2. Centrifuge at $188 \times g$ for 10 min.
3. Resuspend the pellet in 10 ml of depletion buffer and pass the suspension through a cell strainer to remove any clump (*see Note 13*).
4. Count viable cells by trypan blue exclusion.
5. Centrifuge at $188 \times g$ for 10 min.
6. Discard completely the supernatant (remove the last drops using carefully a P200 pipettor without touching the cell pellet).
7. Resuspend the cell pellet in 80 μl of depletion buffer.
8. Add 20 μl of CD56 microbeads.
9. Mix by pipetting carefully and incubate at $4^{\circ}\text{--}8\text{ }^{\circ}\text{C}$ (fridge or cold room) for 15 min. Mix rapidly by flicking during incubation (once). Higher temperature and/or longer incubations lead to nonspecific labeling.
10. Add 2 ml of depletion buffer to wash the cells. Centrifuge at $188 \times g$ for 10 min.
11. Rinse the column with 500 μl of depletion buffer (*see Note 14*).
12. Resuspend the cells in 500 μl of depletion buffer and apply the suspension onto the column.
13. Wash the column with 500 μl three times, once the reservoir is empty (*see Note 15*).
14. Collect the total effluent, containing the CD56– depleted cell fraction.
15. Centrifuge the total effluent at $188 \times g$ for 10 min.
16. Resuspend the cell pellet in 5 ml of M5 medium and proceed to counting of viable cells by trypan blue exclusion.
17. Plate the cells in M5 medium (6000 cells/ cm^2) and put immediately in the incubator at $37\text{ }^{\circ}\text{C}$.

A scheme of the CD56 depletion is shown in Fig. 2.

3.3 Isolation, Propagation, and Freezing of MABs from Murine Skeletal Muscle (mMABs)

At variance with the protocol set for hMABs, a cloning step by limiting dilution has been introduced for mMABs isolation.

Primary polyclonal population, derived from murine skeletal muscle culture under physiological O_2 tension, can be cloned without the support of any feeder layer, on Matrigel-coated plasticware.

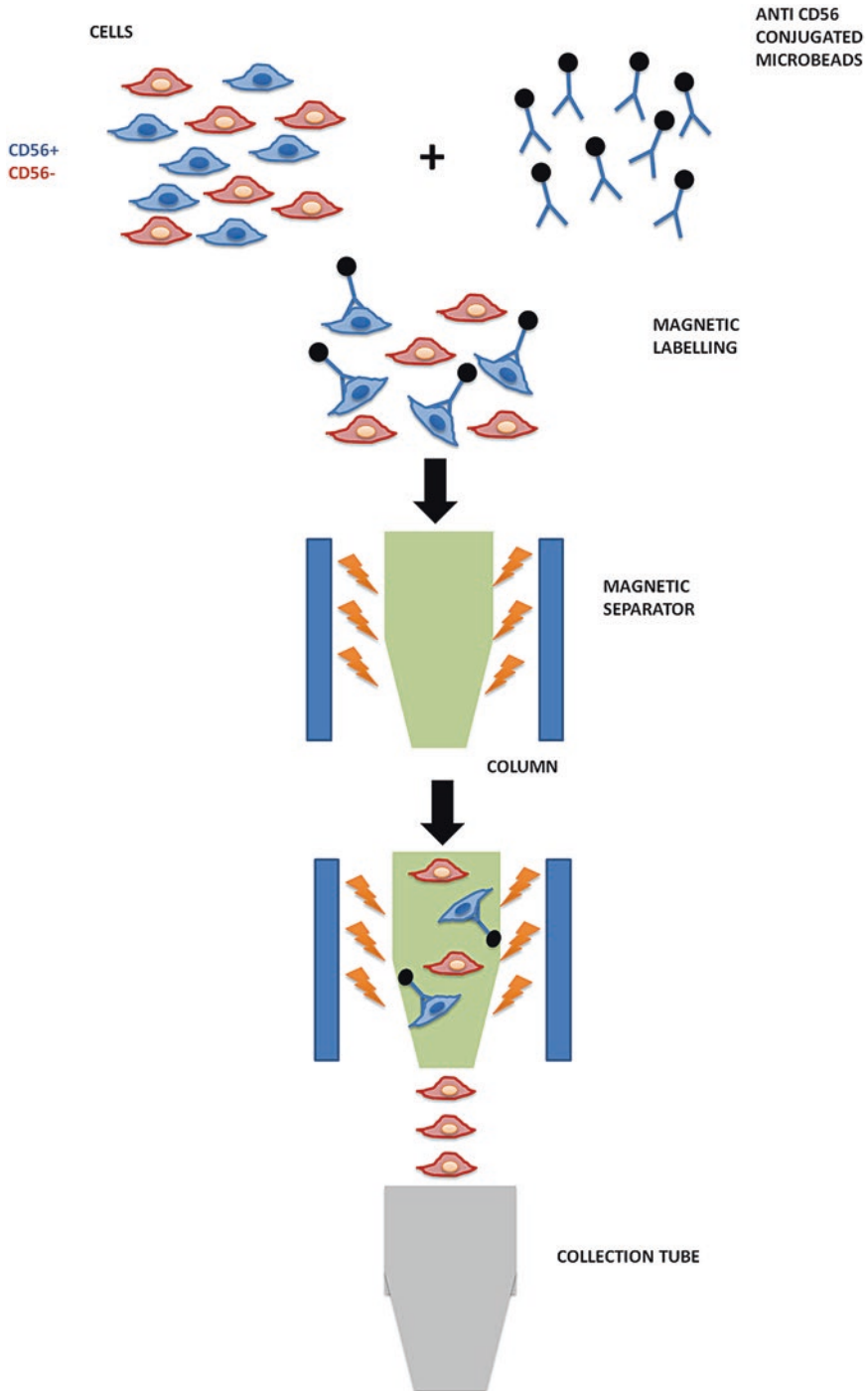


Fig. 2 Depletion of CD56+ cells using magnetic beads. Scheme of the procedure used to separate mesoangioblasts from CD56+ satellite cells

Isolated populations are subsequently screened for PW1/Peg3 to select the ones with highest efficiency in differentiation (*see* Subheading 3.9). On top of physiological O₂ tension, the use of Matrigel, which mimics an extracellular matrix-like microenvironment, represents an additional strategy to improve culture conditions during cloning steps.

Primary culture of murine skeletal muscle is initiated following the same procedures described for human skeletal muscle, (**steps 1–8**), using D20 medium instead of M5.

After 5–7 days, proceed to dissociate the culture as follows:

1. Remove D20 culture medium and carefully rinse twice with 1 ml of CMF-PBS at room temperature, avoiding to touch either the tissue fragments or the surrounding cells.
2. Remove PBS and add 2 ml of collagenase/Dispase solution (*see* recipe in Subheading 2.1) to each dish.
3. Carefully remove cells first and then the tissue fragments by gentle pipetting and mild scraping using a pipettor.
4. Transfer the cells and tissue suspension into 15-ml centrifuge tube.
5. Repeat **steps 2** and **3** three additional times.
6. Add an additional 2 ml of collagenase/Dispase solution directly to centrifuge tube (the digestion of tissue fragments and cells is performed in a final volume of 10 ml for each dish).
7. Incubate 15 min in a 37 °C water bath.
Flick and invert the tube three times during incubation, monitoring the dissociation of tissue.
8. Stop the reaction by adding 3 ml FBS to the tube. Centrifuge at $188 \times g$ for 15 min at room temperature.
9. Discard supernatant and suspend the pellet in 300 μ l of D20 medium.
10. Pipet up and down several times using a 1000- μ l pipettor with filtered tips to disaggregate muscle fragments as much as possible. Let the larger debris sediment for few seconds and transfer the upper more homogeneous cell suspension in a new 15-ml centrifuge tube.
11. Count viable cells by trypan blue exclusion and proceed to cloning (*see* **Note 16**).
12. Dilute cells in D20 medium to obtain 150 ml of each of the following concentrations:
 - 1 cell/ml.
 - 10 cells/ml.
 - 20 cells/ml.
 - 30 cells/ml.

13. For each cell concentration, plate 1 ml/well in three 48-well plates.
14. Prepare a humidified chamber by placing the 48-well plates into a clean plastic box, along with two open 6-cm Petri dishes filled with sterile water. Cover the box with aluminum foil.
15. Place the cultures in a 37 °C, 5 % CO₂, 3 % O₂, and 92 % humidified incubator for at least 1 week.
16. After 1 week, carefully inspect the cultures with a microscope to distinguish the first clone. If clones appear in dishes plated with 1 cell/well, discard dishes plated at higher density.
17. Add 200 µl D20 medium to each well.
18. Passage the clones when the cells have covered at least 50 % of the well surface.
19. At the time of first passage, carefully aspirate the medium and rinse each well with 1 ml of CMF-PBS at room temperature.
20. Add 200 µl of 0.025 % trypsin/EDTA to each well. Incubate 5–10 min at 37 °C, monitoring under microscope for complete detachment of cells.
21. Inactivate trypsin by adding 800 µl D20 medium down the growing surface of each well. Carefully collect all cells.
22. Transfer cells and medium to a 15-ml centrifuge tube and centrifuge 5 min at 188 × *g*, room temperature.
23. Discard supernatant, suspend the pellet in 1 ml fresh D20 medium, and plate in uncoated well of a 24-well plate (*see Note 17*).
24. When 70–80 % confluent, proceed to trypsinization of established mMABs and transfer to uncoated 25-cm² flask.
25. To propagate mMABs, aspirate and discard the medium and rinse with 2 ml of CMF-PBS for 25-cm² flask (5 ml for 75-cm² flasks).
26. Add 1 ml trypsin/EDTA to 25-cm² flask (2 ml for 75-cm² flasks), and incubate 3–5 min at 37 °C. Check under a microscope for complete detachment of cells.
27. Collect the cells with 4 ml of D20 medium (8 ml for 75-cm² flasks).
28. Centrifuge cells 5 min at 227 × *g*, room temperature. Discard supernatant.
29. Suspend the pellet thoroughly in 6–10 ml of D20 medium and dispense 2-ml aliquots of cell suspension into each of three or five flasks (1:3 or 1:5 split, depending upon proliferation rate). Add D20 medium to reach a final volume of 5 ml for 25-cm² flasks (12 ml for 75-cm² flasks). Drag the flasks with a cross movement on the incubator shelf, to ensure homogeneous distribution of cells. Incubate at 37 °C.

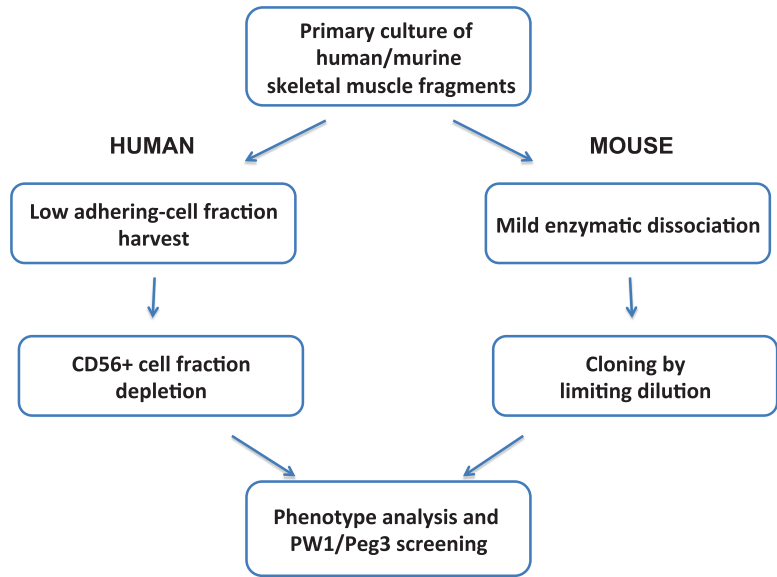


Fig. 3 Schematic framework recapitulating the different steps used for isolation and characterization of human and mouse MABs

Murine MABs are frozen according to the procedure described for human MABs, using D20 instead of M5 medium.

The morphology of mMABs after few passages in culture at the inverted phase contrast microscope is shown in Fig. 1B.

A general scheme on the procedures used for isolation and characterization of human and mouse MABs is shown in Fig. 3.

3.4 AP Staining

The protocols described in this unit lead to the isolation of MAB populations that, at early passage, express AP at a percentage usually $\geq 50\%$.

In case of very low expression percentage ($\leq 10\%$), a FACS step can be introduced [30]. Notably, AP expression physiologically presents high variability and may decrease with passages during in vitro propagation.

1. Remove medium from Petri dishes, rinse the growing surface with 1 ml CMF-PBS.
2. Fix with 1 ml of 4 % PFA 5 min at room temperature.
3. Remove 4 % PFA and rinse with 1 ml CMF-PBS.

Proceed immediately to alkaline phosphatase staining.

Fixed cultures may be stored up to 48 h at 4 °C. If stored, add 0.5 ml CMF-PBS to each dish and seal with Parafilm to avoid drying out and/or contamination.

4. Remove CMF-PBS and add 1 ml of alkaline phosphatase staining solution to each dish.
5. Incubate 2 h at room temperature in the dark.

6. Examine cultures under inverted phase-contrast microscope for a brown cytoplasmic stain, whose intensity is roughly proportional to the level of enzymatic activity.

3.5 Skeletal Muscle Differentiation

Selected MAB populations can be tested for their capability to spontaneously differentiate into skeletal muscle on Matrigel-coated plastics.

1. Plate $0.5\text{--}1 \times 10^5$ m MABS or $1\text{--}2 \times 10^5$ h MABs on Matrigel-coated dishes in 2 ml D20 or M5 medium, respectively. Slight adjustment in cell number/dish may be necessary, due to variability in cell proliferation rate and differentiation efficiency.
2. Incubate overnight at 37 °C, 5 % CO₂.
3. Remove medium and rinse each dish with 1 ml CMF-PBS.
4. Add 2 ml D2 differentiation medium to each dish.
5. Incubate at least 1 week in at 37 °C, 5 % CO₂.
At that time, first myotubes should be evident. A time period of 7–8 days is usually sufficient for mMAB differentiation, while 10–14 days may be necessary for hMABs.
6. Remove medium from Petri dishes and carefully rinse the growing surface with 1 ml CMF-PBS.
7. Fix with 1 ml of 4 % PFA, 5 min at room temperature. Remove 4 % PFA and rinse with 1 ml CMF-PBS. Proceed immediately to immunofluorescence for the expression of sarcomeric MyHC by MF20 antibody (Hybridoma Bank), or store up to 48 h at 4 °C.
8. Calculate percentage of myogenic differentiation as the number of MAB cell nuclei (detected by DAPI) inside myosin positive cells or myotubes divided by the total MAB nuclei multiplied by 100. Examples of human and mouse MAB skeletal muscle differentiation are shown in Fig. 1C, D.

3.6 Induction into Smooth Muscle by TGF-β1 Treatment

1. Plate 5×10^4 cells/3.5-cm Petri dish in 2 ml of medium (D20 for murine, M5 medium for human cells). For each cell line to be tested, plate at least two dishes (one test and one control dish).
2. Incubate overnight in a 37 °C, 5 % CO₂ incubator.
3. Remove medium and rinse each dish with 1 ml CMF-PBS.
4. Add 1.5 ml D2 medium to each dish.
5. Add 1.5 μl TGF-β1 stock solution to test dishes (5 ng/ml final concentration). No addition has to be made to control dishes.
6. Each other day add 1.5 μl TGF-β1 stock solution to test dishes.
7. Check cultures for smooth muscle differentiation, which should be complete after 7–8 days.

8. Remove medium from Petri dishes and carefully rinse the growing surface with 1 ml CMF-PBS.
9. Fix with 1 ml of 4 % PFA 5 min at room temperature.
10. Remove 4 % PFA and rinse with 1 ml CMF-PBS. Proceed immediately to immunofluorescence, or store up to 48 h at 4 °C.
11. Calculate percentage of smooth muscle differentiation as the number of MABs expressing a smooth muscle phenotype (detected by an antibody directed against smooth alpha actin) (Sigma cat. no. A2547) or calponin (Sigma cat.no. C2687) divided by total number of mesoangioblast nuclei multiplied by 100.

Examples of human and mouse MAB smooth muscle differentiation are shown in Fig. 1E, F.

3.7 Induction of Osteoblast Differentiation by BMP2 Treatment

BMP2 treatment results in a strong increase of original alkaline phosphatase activity of MABs, which can be easily detected by AP staining (*see* Subheading 3.4).

Follow **steps 1–4** described for Subheading 3.6.

5. Add 15 µl BMP2 stock solution to each test dish (100 ng/ml final concentration).
No addition has to be made to control dishes.
6. Each other day add 15 µl fresh BMP2 stock solution to test dishes.
7. Assess the cultures for differentiation, which should be complete after 7–8 days.
8. Remove medium from Petri dishes, and carefully rinse the growing surface with 1 ml CMF-PBS.
9. Fix with 1 ml of 4 % PFA 5 min at room temperature.
10. Remove 4 % PFA and rinse with 1 ml CMF-PBS.

Proceed immediately to alkaline phosphatase staining (*see* Subheading 3.4) (*see* **Note 18**).

Examples of human and mouse MAB osteoblast differentiation tested as AP staining are shown in Fig. 1G, H.

3.8 Induction of Adipocyte Differentiation

Follow **steps 1–3** described for Subheading 3.6.

4. Add 1.5 ml of adipogenic induction medium to each test dish.
Add 1.5 ml D2 medium to each control dish.
5. Check cultures for differentiation after 6 to 7–10 days (*see* **Note 19**).

Examples of human and mouse MAB adipocyte differentiation tested as Oil Red O staining are shown in Fig. 1I, J.

3.9 Effect of Different O₂ Tension and Substrate Stiffness on hMAB Growth and Differentiation

Several processes crucial to cell fate determination, such as adhesion, migration, spreading, and differentiation, have been demonstrated to be deeply influenced not only by biochemical but also by physical and mechanical characteristics of surrounding microenvironment.

Among physical parameters, the impact of low oxygen on stemness, proliferation, and differentiation potential has been widely described for several cell type models [31–35]. As far as hMABs are concerned, physiological O₂ tension has been proved to be essential for their isolation, proliferation, and preservation of chromosomal stability, which are crucial parameters for efficient and safe high-scale propagation in cell therapy. Figure 4 shows differences in growth rate of hMABs grown at different conditions of O₂ tension (Fig. 4A–C).

Regarding mechanical parameters, substrate stiffness has been indicated as a primary modulator for stem cell commitment, being capable of directing the differentiation of pluripotent and multipotent stem cells [36–38]. It has been indeed shown how the use of gels with stiffness typical of normal muscle for myoblasts differentiation leads to the formation of myotubes exhibiting highly defined sarcomeric organization [39, 40].

We tested the effect of commercially available elastic substrates on hMAB skeletal muscle differentiation. Specifically, we used a special kind of Petri dish (IBIDI), whose bottom presents a 40- μ m-thick polydimethylsiloxane layer. The layer confers stiffness very close to that of mammalian skeletal muscle (28 KPa, according to Young's Modulus) and, differently from most plastic for cell culture (approximately 300,000 times stiffer), may provide in vitro culture conditions closer to physiology. Figure 4 shows the remarkable effect of this kind of substrate on hMAB orientation and differentiation (Fig. 4D–G).

3.10 PW1/Peg3 as a Screening Molecule to Identify the Best Mesoangioblast Populations

As mentioned in the Abstract, we have recently identified a new marker of MABs, PW1/Peg3, whose expression is shared by all the MABs isolated so far, regardless of the species and the developmental stages [27]. PW1/Peg3 expression strongly correlates with the two main MAB properties, their ability to differentiate in skeletal muscle and to cross the blood vessel wall (Fig. 5). Therefore, we are currently using PW1/Peg3 to screen and identify competent MABs before their use in cell therapy.

3.10.1 Real-Time PCR for Murine and Human PW1/Peg3

1. Thaw all reagents on ice.
2. Prepare the PCR mix; for a final reaction volume of 20 μ l, prepare 15 μ l of premix for each sample. For each well/tube, calculate to add the following components: 10 μ l iTaq Universal or Sso Advanced SYBR Green Supermix (2 \times); 300 nM of each primer; water to 15 μ l. Always consider an additional well to be used as no template control. Prepare the mix in excess.

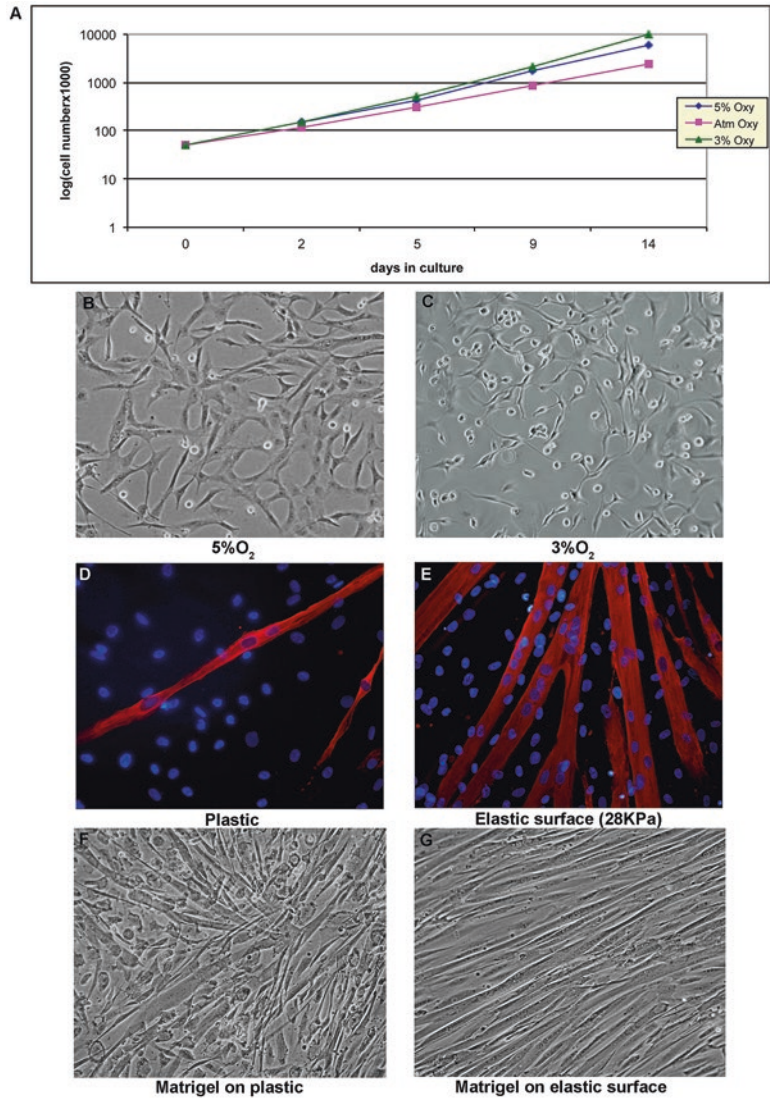


Fig. 4 Effects of stiffness and oxygen levels on MAB cell cultures. **(A)** Example of a growth curve of hMABs in different oxygen conditions (5 %, 3 %, and atmospheric oxygen levels); **(B, C)** appearance of hMABs when cultured at 5 % **(B)** or 3 % **(C)** oxygen; **(D–G)** differentiation properties of hMABs when cultured on different substrates. **(D, E)** Immunofluorescence analysis for all sarcomeric myosins (MyHC, in *red*) of hMABs differentiated on regular plastic **(D)** or on an elastic surface (stiffness 28 KPa) **(E)**. **(F, G)** Phase-contrast images of hMABs differentiated on regular plastic **(F)** or on a Matrigel substrate on elastic surface **(G)**

- Aliquot 15 μ l of premix in each well. High precision while pipetting is necessary (*see Note 20*).
- Load each sample in triplicate. For each well, add 5 μ l of diluted sample cDNA. Mix by pipetting up and down. High precision while pipetting is necessary (*see Note 21*).

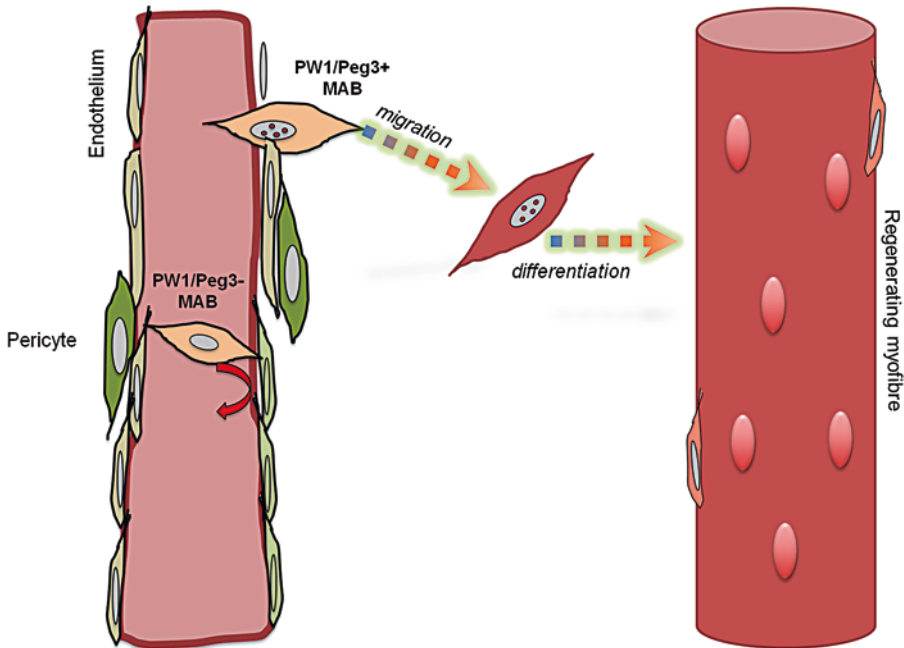


Fig. 5 Scheme exemplifying the importance of PW1/Peg3 activity for both human and mouse MAB main properties: skeletal muscle differentiation and vessel transmigration

5. Close tubes/plate and load in the Real-Time PCR Thermal Cycler.
6. Apply the following thermal protocols:
 - (a) For murine PW1: 98 °C, 30 s; (95 °C, 3 s; 54 °C 30 s) for 40 cycles; melt curve (65–95 °C, 0.5 °C increments at 2 s/step).
 - (b) For human Peg3: 98 °C, 30 s; (98 °C, 3 s; 60 °C 10 s; 72 °C 15 s) for 40 cycles; melt curve (65–95 °C, 0.5 °C increments at 2 s/step).
7. Normalize each sample to the housekeeping gene (GAPDH) and calculate expression levels using the comparative threshold cycle [41] method (*see Note 22*).

3.10.2 Immuno-fluorescence for Murine PW1

1. Rapidly wash cells with PBS to eliminate cell culture medium excess.
2. Fix cells with 4 % paraformaldehyde in PBS, for 10 min at 4 °C.
3. Eliminate paraformaldehyde and wash three times with PBS (5 min each at RT).
4. Permeabilize cells with precooled methanol at –20 °C for 6 min.
5. Block with blocking solution for 3 h at RT.

6. Incubate cells overnight at 4 °C with rabbit anti-mouse PW1 primary antibody (1:3000) diluted in blocking solution (*see Note 2*).
7. The day after, wash cells three times (15 min each at RT) with 0.1 % BSA in PBS.
8. Wash again in 5 % BSA in PBS for 15 min at RT.
9. Incubate cells with secondary antibody (we use Jackson Laboratories Antibodies, 1:500), together with DAPI (1:500, Sigma) diluted in blocking solution for 2 h at RT (*see Note 23*).
10. Rinse cells with 0.1 % BSA in PBS for three times (15 min each at RT).
11. Rinse cells with 5 % BSA in PBS for 15 min at RT.
12. Mount cells with fluorescence mounting medium and cover with a coverslip.
13. Watch under fluorescence microscope (*see Note 24*).

3.10.3 Western Blot for Murine PW1

1. Proceed with protein extraction. Lyse samples in RIPA lysis buffer, scraping cells from the Petri dish and transferring the sample to a 1.5-ml tube. Let samples on ice for 30 min, vortexing every 10 min. Centrifuge for 10 min at 12000×g, 4 °C. Transfer protein-enriched supernatant to a new tube.
2. For every sample, load 30 µg of protein extracts along with 2× loading buffer (pre-boil samples for 5 min at 95 °C) on a 6 % acrylamide gel.
3. Perform the run in running buffer, at constant voltage (100 V).
4. Transfer the gel on nitrocellulose membrane using the iBlot Dry Blotting System (Life Technologies), for 10 min.
5. Block the nitrocellulose membrane with 5 % skimmed milk, 0.05 % Tween 20 in PBS for 1 h at RT on a rotating platform.
6. Incubate overnight at 4 °C on a rotating platform, with rabbit anti-mouse PW1 primary antibody (1:10,000) diluted in 5 % skimmed milk, 0.05 % Tween 20 in PBS.
7. The day after, wash four times (10 min each) with T-PBS at RT while shaking.
8. Incubate with HRP-conjugated goat anti-rabbit secondary antibody (1:10,000) diluted in 5 % skimmed milk in T-PBS for 45 min at RT while shaking.
9. Repeat the washes as described in **step 7**.
10. Incubate with ECL chemiluminescence reagents.
11. Expose the membrane to an X-ray film and develop or acquire signal with the digital chemiluminescence imaging system (*see Note 25*).

4 Notes

1. Both human and murine skeletal muscle fragments can be stored in D20 medium, at 4 °C, up to 24 h before being processed.
2. A reliable antibody that recognizes human Peg3 is not available commercially. The one used for mouse PW1/Peg3 has been described in the following studies [27, 28]. This group has recently generated a monoclonal antibody for the human form of Peg3 as well. For further information, please contact Dr. Sassoon at david.a.sassoon@gmail.com.
3. The use of a stereomicroscope may help for best selection in case of damaged tissue (especially for samples obtained from donors undergoing post-traumatic surgery). Try to identify portions of interstitial tissue containing small vessels. Remove as much adipose tissue as possible (since its presence may delay hMAB proliferation). Proceed with sharp and neat cuts (avoid any shearing/dissociation of the tissue, which may lower the yield of hMAB isolation).
4. Due to small volume of medium used at early stages of culture initiation, it is critical to prevent its evaporation (since this will cause tissue suffering and cell death), setting a humidified chamber. This can be obtained by placing the dish containing the dissected fragments into a 15-cm Petri dish, along with an open Petri dish filled with sterile distilled water.
5. Early phase of hMAB derivation requires a constant O₂/CO₂ tension and humidity level. Therefore use a dedicated incubator or at least a rarely opened incubator.
6. Gentle pipetting may help to detach the weakly adhering cells around the explants. Plate detached cells in uncoated Petri dish (same size of dish used for primary culture assembling). Incubate 1–3 days. At this point, the floating population of hMABs should be easily distinguishable. Transfer medium and floating cells to a new dish or 25-cm² tissue culture flask. Discard the primary mixed population of adherent cells.
7. hMABs cultured in vitro in M5 medium grow as floating cells when in duplicative phase. Consequently the floating fraction represents a stable and renewable source of uncommitted hMABs. When transferred to untreated plasticware, hMABs can adhere, but a floating/low-adhering fraction of dividing cells should always be present in healthy cultures, particularly in early stage populations. The recovery of an early floating/low-adhering fraction is a crucial step for proper hMAB isolation. The coating of plasticware is not necessary after primary culture step.

8. hMABs are very sensitive to trypsin. If cells are healthy, their detachment should be very quick and complete at room temp. The day after trypsinization, the floating population may be reduced. Normally, this fraction should start to expand again after 48 h.
9. When MABs have been expanded to 25-cm² tissue culture flasks (approximately 500,000 cells/flask), proceed to:
 - Karyotype analysis.
 - Phenotype analysis and eventual CD56⁺ cell fraction depletion.
 - PW1/Peg3 screening.
 - Freezing of early passage cells, for backup and further propagation.
10. hMABs can be expanded up to 20 passages under 3 % O₂ tension. At pre-senescence a strong reduction in the floating population of cells is observed, in addition to the presence of large, flat, or elongated, vacuolated cells. Late passage hMABs cell lines can be easily screened for the presence of senescent cells by assays based on histochemical stain for β -galactosidase activity at pH 6 (senescence histochemical staining kit, SIGMA cat. No. CS0030).
11. At moment of freezing hMABs should be in active proliferation (a floating fraction should be evident), and adherent fraction of cells should be at 70–80 % of confluence.
12. Upon thawing, which has to be performed quickly in a 37 °C water bath, transfer the vial content into a 15-ml centrifuge tube containing 5 ml of pre-warmed M5 culture medium; centrifuge 5 min at 227 × *g*, room temperature; discard supernatant to remove DMSO; and resuspend cells in M5 medium and plate.
13. In order to obtain an efficient depletion, it is very important to obtain a single cell suspension. Pre-wet the cell strainer with 2 ml of depletion buffer before use, to prevent the attachment of cells to the filter membrane.
14. During the centrifugation of the sample, insert the LD column in the magnetic separator, and put the separator on its stand and a suitable collection tube under the column. All the depletion procedure has to be performed under a flow hood.
15. To increase purity, the procedure can be repeated with a new column, without any effect on cells viability.
16. In parallel to cloning, a small aliquot (30 μ l) of total cell suspension should be plated in a single well of 48 Matrigel-coated multiwell to check for cell survival rate. It is recommendable to expand and freeze an aliquot of polyclonal mix for backup and further analysis.

17. From this step on, no Matrigel coating will be necessary, but particular attention will have to be paid to the density of cells. Until the third/fourth passage, cells must be grown at high density and must be split when confluent into progressively larger wells (from 48- to 24- to 12- to 6-well plates). This phase is the most critical for mesoangioblast derivation; in fact, many clones may differentiate or go to senescence and/or stasis; if culture conditions are inadequate, all clones may be lost at this stage. The successful, continuously proliferating clones usually represent a small percentage of all subcultured clones (from 5 to 10 %). A clone can be considered “established” if cells proliferate at a regular rate (approximately 12 h doubling time) and maintain a typical morphology. Once established, all clones (or at least a significant number) need to be propagated and characterized (phenotype analysis, AP staining, PW1/Peg3 screening).
18. For a more rigorous test of osteoblast differentiation, *in vitro* formation of von Kossa positive, calcified nodules (Chaplin and Grace, 1975) should be characterized.
19. Differentiation into adipocytes is morphologically easy to detect. It is characterized by the presence of gradually enlarging, translucent vacuoles in the cytoplasm of a percentage of cells (up to 60–70 %). The presence of lipid content in these vacuoles must be confirmed by appropriate staining (Oil Red O staining).
20. To ensure maximal precision while pipetting the PCR mix and avoid bubble formation, we usually apply the repetitive pipetting technique. This technique consists in overloading the tip while aspirating the mix, pressing the operating button to the second stop, and dispensing the mix by pressing the operating button to the first stop. When using this pipetting technique, a single tip can be used to aliquot the PCR mix in all the wells/tubes used.
21. Change tip for every well/tube loaded with samples. While dispensing cDNA, mix several times by pipetting up and down. To avoid bubble formation, mix and dispense samples pressing the operating button just to the first stop.
22. It is always necessary to assess primers efficiency in the temperature and condition used before proceeding with the analysis.
23. To avoid the waste of antibody, delimit the immunofluorescence area with a liquid-blocker pen (we use PAP pen, Sigma). Use the pen to draw a hydrophobic circle around the area of interest. This will limit the amount of antibody to be used (typically 200 μ l for 35-mm petri dishes).

24. The immunostaining for PW1 in murine MABs is clearly nuclear, with nucleolus exclusion.
25. Typically, incubation with PW1 antibody results in a predominant, aspecific band around 100 kDa and a correct band around 250 kDa.

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