State of the art procedures for the isolation and characterization of mesoangioblasts

Nefele Giarratana[1,2], Filippo Conti[1,3], Flavio Ronzoni[3,4§] and Maurilio Sampaolesi[1,3§*]

[1]Translational Cardiomyology, Stem Cell Research Institute, Catholic University of Leuven, Herestraat 49 B-3000 Leuven, Belgium;
[2]Stem Cell Laboratory, Department of Pathophysiology and Transplantation, University of Milan, Unit of Neurology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Centro Dino Ferrari, Milan, Italy;
[3]Human Anatomy, University of Pavia, Via Forlanini 8, 27100 Pavia, Italy;
[4]Department of Biomedical Sciences, Humanitas University, Rozzano, Italy.

§ These authors contributed equally.

*Address correspondence to:
Maurilio Sampaolesi Ph.D.
Stem Cell Research Institute
49 Herestraat, B-3000 Leuven, Belgium.
Tel: +32-(0)163-30295; Fax: +32-(0)163-30294
e-mail: maurilio.sampaolesi@kuleuven.be

SUMMARY

Adult skeletal muscle is a dynamic tissue able to regenerate quite efficiently thanks to the presence of a stem cell machinery. Besides the quiescent satellite cells that are activated upon injury or paracrine factors other stem cells are described to be directly or indirectly involved in adult myogenesis. Mesoangioblasts (MABs) are vessel-associated stem cells originally isolated form embryonic dorsal aorta and then from adult muscle interstitia expressing pericyte markers. Recently, adult MABs entered clinical trials for the treatment of Duchenne muscular dystrophy and the transcriptome of human fetal MABs has been described. In addition, single cell RNA-seq analyses provide novel information on adult murine MABs and more in general in interstitial muscle stem cells. This chapter provides the state-of-the-art techniques to isolate and characterize murine MABs, fetal and adult human MABs.

Key words: human adult and fetal stem cells, mesoangioblasts, skeletal muscle regeneration, stem cell culture, FACS, single cell RNA-seq, transcriptome

1. Introduction

The scientific community is still debating on the intrinsic regeneration ability of skeletal muscles and there is a large consensus that besides satellite cells, known as skeletal muscle quiescent progenitors (*1, 2*), other adult stem cell populations contribute to adult myogenesis (*3*). In injured skeletal muscles, several chemokines, growth factors and eventually recombinant proteins (*4, 5*), play a crucial role in the stem cell machinery. Although satellite cells are the main players in skeletal muscle development and repair, other local progenitors, including mesoangioblasts (MABs) have been showed to directly contribute to muscle repair (*6-12*). MABs were originally isolated from murine dorsal aorta and subsequently identified in murine and human adult skeletal muscles associated with small interstitial vessels. MABs still retain the ability to differentiate in mesodermal cell lineages (*13*), including osteogenic, adipogenic and chondrogenic cell types, and they can extensively expanded in vitro since they express the clonogenic marker CD146 (see Table 1). In muscle degenerative disease animal models, several studies have shown the intrinsic capacity of MABs to contribute to muscle regeneration (*14-18*). Recently it has been demonstrated

that MABs derived from fetal tissues show high plasticity and elevate differentiation capabilities (*19*). In particular, transcriptional profiles of MABs derived from aorta, atrial, ventricular, and skeletal muscles of fetuses revealed that each subset of MABs displayed a set of differentially expressed genes, which seem to reflect their distinct tissue derivations. The differential transcription profiles of MABs also correlated with the inherent myogenic differentiation properties of each tissue type. Moreover, while differentially expressed gene profiles demonstrated a global opposite set of upregulated and downregulated genes between skeletal and cardiac muscle MABs, the aorta MABs displayed an intermediate profile. Moreover, both fetal and adult MABs can be easily transduced with lentiviruses (*20*).

In this book chapter we illustrate the current protocols for isolation, expansion, characterization and freezing procedures of MABs from adult murine skeletal muscles and from human adult and fetal muscle biopsies (*21, 22*). Fluorescence-activated cell sorter (FACS) techniques are not only crucial for the isolation and characterization of fetal and adult MABs, but also for the more innovative single-cell OMICS analyses, including single cell RNA sequencing (scRNA-seq) as reported. A full protocol complete with procedures for collagen-based coating of tissue culture surfaces for MABs is also provided. Since MABs have been recently tested in clinical trials (*15*), we also provide a method to test their cell fusion potential by means of C2C12 cell coculture experiments. Indeed, C2C12 cells are considered as the gold standard for myogenic cell lines, since activate satellite cells tend to differentiate quickly and it is not possible to keep them in culture in undifferentiated state. We also describe various cell differentiation methods, including spontaneous myogenic differentiation, induction of smooth muscle cells, osteocytes, adipocytes and chondrocytes. It is important to note that basic animal handling, dissection, and tissue culture skills are mandatory for successful attempts in order to obtain and expand MABs *in vitro*. It is also necessary to have basic knowledge in histochemistry, biochemistry, and molecular biology for the successful characterization of MABs. Importantly, sterile conditions in either Class II biohazard flow hoods are recommended for human materials and approvals from local Institutional Ethics Committee and patient informed consent are needed.

Finally, note-taking is a crucial point of the troubleshooting process thus, we provide a list of notes to keep the process of troubleshooting as easy as possible especially for beginners.

2. Materials

2.1 *Basic Materials*

- Skeletal muscle biopsies from murine or human samples (see Methods)
- C2C12 myogenic cell line (ATCC # CRL-1772)
- Sterile rounded-edge disposable scalpels
- Sterile curved forceps
- Sterile sharp-edged straight forceps
- 3.5-, 6-, 10 cm, Petri dishes (Nunc, Denmark)
- Calf skin collagen (Sigma Aldrich, USA)
- Suitable polypropylene tubes, with and without cell filter
- Culture-grade water (Thermo Scientific, USA)
- Sterile Phosphate-buffered saline (PBS) Ca^{2+}/ Mg^{2+}-free (Gibco, USA)
- Sterile TrypLE Express Trypsin (Gibco, USA)
- Glacial acetic acid (Sigma Aldrich, USA)
- Cell incubator set at 5% CO_2, 5% O_2, 90% N_2
- Phycoerythrin-conjugated monoclonal anti-human/mouse AP, clone B4-78 (R&D, USA)
- 7-ADD dead or alive markers (Thermo Fischer scientific, US)
- Cryobox (Nalgene, USA)

2.2 *Media*

- Collagen solution (100ml): Dissolve by stirring 0.005mg/ml of calf skin collagen in glacial acetic acid (i.e. 100 mg/20 ml) overnight at room temperature. Carefully mix and add 20% of the acid collagen solution to culture-grade water (i.e 80 ml). Filter through a 0.22 µm membrane to ensure sterility (Stericup, Millipore #C3240). Store at 4°C.
- Growth medium for murine MABs (DMEM20 medium; 250ml): DMEM high glucose (Gibco, USA), supplemented with 20% sterile heat-inactivated Fetal Bovine Serum (FBS), 1% penicillin/streptomycin solution (100 units), 2 mM glutamine, 1 mM sodium pyruvate and 1% non-essential amino acid solution. Filter through a 0.22 µm membrane to ensure sterility (Stericup, Millipore #C3240). Store at 4°C for up to 3 weeks.

- Growth medium for human MABs (IMDM15 medium; 250 ml): IMDM (Sigma, USA), supplemented with 15% sterile heat-inactivated FBS, 1% penicillin/streptomycin solution (100 units), 2 mM glutamine, 1% non-essential amino acid solution, 0.1 mM 2-mercaptoethanol, 1% of 100X Insulin-Transferrin-Selenium and 1.25 μg human bFGF (Peprotech, USA). Filter through a 0.22 μm membrane to ensure sterility (Stericup, Millipore #C3240). When supplemented with bFGF store at 4°C for up to 1 week, else store for longer and add bFGF freshly upon using (second option is recommended).
- Growth medium for human fetal MABs (MegaCell DMEM medium; 250 ml): MegaCell DMEM (Sigma, USA) supplemented with 5% fetal bovine serum (FBS, Lonza BioWhittaker), 5 ng/mL of human bFGF (Peprotech, USA) freshly added, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% non-essential amino acid solution, and 1% penicillin/ streptomycin (all from Gibco, Invitrogen). Filter through a 0.22 μm membrane to ensure sterility (Stericup, Millipore #C3240).
- C2C12 myoblast cell growth medium (DMEM10 medium; 250 ml): DMEM high glucose supplemented with 10% sterile heat-inactivated FBS, 1% penicillin/streptomycin solution, 1 mM sodium pyruvate (all reagents by Gibco, USA). Filter through a 0.22 μm membrane to ensure sterility (Stericup, Millipore #C3240). Store at 4°C for up to 2 weeks.
- Freezing medium (FM; 50 ml): heat-inactivated FBS (Gibco, USA) supplemented with 10% Hybri-MAX® DMSO (Sigma, USA). Filter through a 0.22 μm membrane to ensure sterility (Steriflip, Millipore #C3238). Store at 4°C for up to 4 weeks. Keep cold until use.
- Spontaneous differentiation medium (DM; 250 ml): DMEM high glucose supplemented with 2% sterile heat-inactivated Horse Serum (HS), 1% penicillin/streptomycin solution, 2 mM glutamine and 1 mM sodium pyruvate (all reagents by Gibco, USA). Filter through a 0.22 μm membrane to ensure sterility (Stericup, Millipore #C3240). Store at 4°C for up to 4 weeks.
- Smooth muscle differentiation medium (SMM medium; 250 ml): DMEM high glucose supplemented with 2% sterile heat-inactivated HS, 1% penicillin/streptomycin solution, 2 mM glutamine, 1 mM sodium pyruvate (all reagents by Gibco, USA) and 1.25 μg TGFβ (Peprotech, USA). Filter through a 0.22 μm membrane to ensure sterility (Stericup,

Millipore #C3240). When supplemented with TGFβ store at 4° for up to 1 week, else store for longer and add TGFβ fresh upon using (second option strongly recommended).

- Osteogenic differentiation medium (OM; 250 ml): αMEM basal medium supplemented with 10% sterile heat-inactivated FBS (Gibco, USA), 0.1 µM dexamethasone, 2 mM glutamine, 50 µM ascorbic acid, 10 µM 2-glycerophosphate, 1% penicillin/streptomycin (all reagents by Lonza, USA). Filter through a 0.22 µm membrane to ensure sterility (Stericup, Millipore #C3240). Store at 4°C in the dark for 4 weeks.
- Adipogenic differentiation medium (AD medium): we recommend the use of StemPro Adipogenesis differentiation kit (Invitrogen).
- Chondrogenic differentiation medium (CD medium): we suggest StemPro Chondrogenesis differentiation kit (Thermofisher).

3. Methods

3.1 Adult MAB isolation

Murine and human MABs can be isolated from hindlimb skeletal muscles of adult animals (see Note 1) and from small muscle biopsies respectively. Adult muscle fragments can be stored in DMEM20 medium up to one day at 4°C before proceeding further.

Positive cells for CD140a, CD140b and Alkaline Phosphatase and lineage negative (lin^-) for endothelial and hematopoietic markers ($CD31^-$, $CD45^-$ and $Ter119^-$) can be sorted out from the murine bulk cell population (see Table 1). Similarly, lin^- cells positive for CD140a, CD140b and Alkaline Phosphatase and can be sorted out from the human bulk cell population. In order to avoid satellite cell contaminations, anti-CD56 and/or anti-alpha-7 integrin (Itga7) can be included in the list of lin^- Abs.

3.2 Human fetal MAB isolation

Fetal tissue samples could be obtained from aborted material of gestational age normally between 9.5-13 weeks donated to research under informed consent.

Human fetal MABs (hfMABs) can be isolated from aorta, cardiac and skeletal muscle fragments as previously described (*23*). Rinse skeletal muscle fragments in phosphate-buffered saline (PBS) (w/o Ca2+ Mg 2+), cut into small pieces (1–2 mm diameter) and transfer to a Petri dishes

previously coated with type I collagen (Sigma-Aldrich) (see Note 2, 3). The medium is composed by MegaCell DMEM (Sigma-Aldrich) supplemented with 5% fetal bovine serum (FBS, Lonza BioWhittaker), 5 ng/ml basic fibroblast growth factor (bFGF, R&D Systems), 2 mM L-glutamine, 0.1 mM ß-mercaptoethanol, 1% nonessential amino acids, and 1% penicillin/streptomycin (all from Gibco, Invitrogen). Culture skeletal muscle fragments approximately for 7–10 days and after the initial outgrowth of fibroblast-like cells, round-shaped and reflective cells could be observed on top of them (see Note 4). Seed hfMAB cultures at $5x10^3$ cells per cm^2 in a 5.5% CO2 humidified incubator in hypoxic conditions (5% O2) and split cells every 2–3 days

3.3 Murine adult MAB isolation by fluorescence-activated cell sorter (FACS)

- As the second incubation starts, monitor the fragments daily: add 500 ml of fresh DMEM20 and check the extent of cell spread from the biopsies. If the medium acidifies, gently remove it and change it with fresh DMEM20. Once the cell layer spreads for approximately 1 cm from each fragment (see Figure 3), immediately proceed to the next step (see Note 4).
- Firstly, place the muscles from each mouse in a 10 cm dish and rinse them with 5 ml PBS to wash away the blood. Using separated dishes for each muscle is recommended for separated isolation from different muscles.
- Move the muscles onto a clean 10 cm dish. After checking for and removing any fibrous or fat tissue that may be left, use a sterile, round-shaped scalpel to dissect the muscle into fragments measuring about 2 mm^2.
- With the help of a sterile, curved forceps, transfer the fragments obtained from each muscle onto a 3.5 cm, collagen-coated dish (see Note 2). The distance between the samples must be kept constant (to ensure optimal results, 10 fragments should be placed on each dish, at 8-9 mm from one another).
- Delicately put 100 ml of pre-warmed DMEM20 on top of every muscle fragment, then start an 18-24 hours incubation in a humidified incubator (37°C, 5% CO_2/5% O_2) (see Note 3).
- After the incubation, use 1.5 ml of DMEM20 for each dish to cover the fragments. To avoid detachment of the samples, add the medium slowly, against the side of the dish. Clean the dished from any fragment that did not attach by using a sterile, curved forceps. Incubate again, for at least 72 hours (37°C, 5% CO_2/5% O_2).

- As the second incubation starts, monitor the fragments daily: add 500 ml of fresh DMEM20 and check the extent of cell spread from the biopsies. If the medium acidifies, gently remove it and replace it with fresh DMEM20. Once the cell layer spreads for approximately 1 cm from each fragment (see Figure 3), immediately proceed to the next step (see Note 4).
- Using a sterile, sharp-edged forceps, gently remove the fragments from the dish, collect the medium in a 15- or 50-ml tube, then wash with PBS. Do not discard the medium since some cells might be present in suspension.
- To detach the cells, add 600 μl of pre-warmed trypsin to the dish and let it act for 2-3 minutes at 37°C, gently tapping on the sides of the dish to help detachment. Once the cells are suspended, add 1 ml of DMEM20 to the dish and carefully collect all the cells into a 15- or 50-ml tube (the same used to collect the medium) (rinsing the dish by gently pipetting the suspension is recommended to avoid losing cells). Form cells pellets by centrifuging the samples for 5 minutes (500 g, room temperature).
- Add a suitable volume of DMEM20 to the tube and homogeneously resuspend the cell pellet (no clumps should be present), then proceed to count the cells.
- At this point, is generally better to immediately proceed to the next step, avoiding re-plating the cells. However, if the number of cells is limited ($< 5\times10^5$), re-plate them onto collagen-coated 6-multiwell-plates and wait until the confluence reaches 85-90% before proceeding to the next step.
- Prepare sterile, FACS-suitable, capped polystyrene tubes and add:
  - 10^5 cells for the blank sample
  - The remaining cells for the sorting sample ($> 1\times10^6$ is suggested)
- Centrifuge the samples for 5 minutes at 500 g at room temperature, discard the supernatant and re-suspend the cells pellets either by gently vortexing or by pipetting. For the blank sample use 200 μl of PBS, for the sorting sample, instead, use 48 μl of PBS together with 2 μl (1:25) of the suitable Alkaline-Phosphatase (AP) FACS antibody (see also Note 5).
- Incubate for 30 minutes, keeping the samples covered from the light and on ice.
- After the incubation, centrifuge for 5 minutes at 500 g at room temperature, discard the supernatant, add 200 μl of PBS and vortex gently to homogenously wash the cells.
- Repeat this last wash step once.

- Move the samples in a 5 ml polystyrene tube while filtering the cells (place a filter on top of the tube or use a filter top tip), then proceed to sort the cells: MABs will stain positive for AP (see Figure 3). Additionally, it is suggested to stain the cells with 7-AAD dead or alive markers (1:25) just before sorting.
- Collect the sorted MABs in sterile, FACS-suitable polypropylene tubes, previously filled with 500 µl of DMEM20. Supplement the medium with penicillin (1:20) and streptomycin (100 units) to prevent contaminations (see Note 6).
- Centrifuge the sorted cells for 5 minutes at 500 g at room temperature, discard the supernatant and carefully resuspend them in an appropriate volume of DMEM20, and finally plate them onto a suitable surface (see Note 7).
- Culture and expand the obtained MABs on collagen-coated, multi-well plates, using DMEM20 and keeping them in a humidified incubator (37°C, 5% CO_2/5% O_2).
- Passaging cells is recommended with a confluence of 85-90%, at a 1:5 ratio. MABs can maintain their proliferation/differentiation capacity for 20-25 passages. In later passages, MABs generally undergo senescence, or anyway a loss of potency can be observed.

3.4 Human adult MAB (hMAB) isolation by FACS

- To harvest the cells, follow the same protocol as for murine MABs (see above, Paragraph 3.3). Replace DMEM20 with IMDM15 medium (see Note 8).
- Culture and expand adult human MABs on collagen-coated, multi-well plates, using IMDM15 and keeping them in a humidified incubator (37°C, 5% CO_2/5% O_2).
- Passaging cells is recommended with a confluence of 80%, at a 1:3 ratio (see Note 9). Human adult MABs can maintain their proliferation/differentiation capacity for about 20 passages. In later passages, adult human MABs generally start showing signs of senescence and apoptosis.

3.5 Human fetal MAB (hfMAB) isolation by FACS

- Detach the cells with 0.05% trypsin-EDTA and rinse with PBS containing 3% FBS.
- Incubate around 2-3x10^6 cells for 20 min at 4°C in the dark with specific conjugated mouse anti-human antibodies or isotype controls (1µg/ml): PE-Cy7-conjugated anti-alkaline phosphatase (AP) (R&D Systems), APC-conjugated anti-CD13 (e-Bioscience), FITC-conjugated anti-CD90 (BD-Pharmingen), PE-Cy7-conjugated anti-CD146 (BD-Pharmingen),

AlexaFluor647-conjugated anti-NG2 and AlexaFluor488 anti-CD56 (BD-Pharmingen) (see Table 1).

3.6 MAB batches long-term storage

- After expansion, remove medium, wash with PBS, cover the cell layer with a proper amount of trypsin.
- Incubate 5 min at 37°C in a 5% CO_2/ 5% O_2 humidified incubator. Add the same amount of growing medium to block trypsin reaction, carefully collect the cells in a 15 ml tube. Count the viable cells and spin down 5 min at 400 g at room temperature.
- Resuspend in a suitable amount of FM medium (1ml/ 2 x 10^6 cells) and pipet 1 ml of cell suspension per cryovial.
- Incubate the cryovials in isopropanol-containing cryobox overnight at -80°C. After 24 hours, transfer the vials into -150°C freezers or liquid N_2 tanks for long-term storage.

3.7 Murine adult MAB isolation by FACS for single cell RNA sequencing (scRNA-seq)

- Single-cell sort LIN- muscle cells by FACS in 96 well plates (4titude, #4ti-0960). Each well contained 0.4 per cent Triton X-100 in RNase-free water supplemented with 10 mM biotinylated Oligo-dT (IDT), 10 mM dNTPs (Thermo Scientific #R0181) and 0.5 U/µl RNase inhibitor (Takara Clontech #2313), for a total volume of 4 µl lysis buffer.
- cDNA libraries were generated based on the Smart-seq2 protocol (*24*, *25*).
- Briefly, incubate lysed cells at 72 °C for 3 min and amplify cDNA via a 22 cycles PCR. Amplification is done with KAPA HIFI Hot Start ReadyMix (Roche #07958919001) and purification by magnetic beads (CleanNA #CPCR).
- Assess quantity and quality of cDNA with a Qubit fluorometer (Thermo Scientific) and Agilent 2100 BioAnalyzer with a high-sensitivity chip, respectively.
- Make library preparation with the Nextera XT library prep and index kit (Illumina #FC-131-1096 and #FC-131-2001). Tagment 100 pg of cDNA by transposase Tn5 and amplify it with dual-index primers (i7 and i5, Illumina, 14 cycles). Mix the reagents together by the Echo 555 (Labcyte) and purify the pooled Nextera XT libraries. Pool together the single-cell libraries (384 in this experiment) and sequence single-end 50 bp on a single lane of a Hiseq2500 or HiSeq4000 (Illumina).

- All results related to scRNA-seq (Figure 1 and 2) are based on freshly isolated muscle cells from C57Bl6 mice, experiments are performed on the same day.

3.8 scRNA-seq analysis

- Analysis of SmartSeq2 scRNA-seq data can be performed with the Seurat (26) R package (version 3.0.1).
- For importing data into SEURAT, make the raw counts previously gathered (*24*) compatible by transforming ENSEMBL# to gene Symbol.
- Filter cells containing a high content of mitochondrial genes and a high content of ERCC's (SEURAT QC).
- Further analyze the remaining cells for their expression value scaling and normalization.
- PCA and UMAP dimensionality reductions and clustering were performed. The expression values were renormalized, rescaled and re-clustered and cells were manually annotated based on their differentially expressed genes (Figure 1 A-C).
- Identification and analysis of differentially expressed gene markers representative for each cluster are shown by violin plots. Marker gene median is represented and every dot within the violin indicates one single cell (Figure 2 A).

3.9 Collagen-coating

- Add collagen solution until the bottom is homogeneously covered. Incubate 5 min at room temperature, remove the collagen solution and dry the dish out. Incubate the dish at 37°C overnight in a sterile oven.
- After 24 hours, wash the surface at least 3 times with PBS. Before seeding cells, ensure the correct pH by covering the bottom with a RedPhenol-containing medium. If the medium turns yellow, wash again with PBS.

3.10 Cell fusion potential: MABs and C2C12 cell cocultures

- Expand murine C2C12 myoblasts in DMEM10 medium at 37°C in a 5% CO_2/ 5% O_2 humidified incubator, splitting 1:6 upon 70% confluence. Change DMEM10 medium daily and avoid myotube formation.
- At day 0 of differentiation, start the cocultures seeding together C2C12 myoblasts and murine or rat MABs with the ration of 1:1 so that after 24 hours cells will be 80-85% confluent ($2x10^4$

in each well of collagen coated 12 mw). Incubate at 37°C with DMEM20 medium. After 24 hours, remove medium, wash with PBS, add myogenic differentiation medium and incubate.

- Refresh DM medium every 2-3 days, until appearance of myotubes (usually after approximately 5-7 days) and proceed to analyses (see Note 10).

3.11 MAB smooth muscle induction

- Expand MABs, either with IMDM15 or with DMEM20 medium for human and murine adult cells respectively and MegaCell DMEM medium for hfMABs, at 37°C in a 5% CO_2/ 5% O_2 humidified incubator.
- To induce smooth muscle differentiation, plate either ~5.10^3 cells/ cm^2 for murine MABs, or ~$1x10^4$ cells/cm^2 for human MABs in collagen-coated plates, incubated at 37°C with either murine or human growth medium. Day -1 of differentiation.
- After 24 hours, remove medium, wash with PBS, add SMM medium and incubate for 7-8 days, changing the medium every 2 days. Proceed to analyses (see Note 11).

3.12 MAB osteogenic induction

- Expand MABs, either with IMDM15 or with DMEM20 medium for human and murine adult cells respectively and MegaCell DMEM medium for hfMABs, at 37°C in a 5% CO_2/ 5% O_2 humidified incubator.
- Upon 100% confluence, remove medium, wash with PBS, add OM medium and incubate.
- Change OM medium every 4 days for 2-3 weeks and proceed to analyses (see Note 12 and Figure 3).

3.13 MAB adipogenic induction

- Expand MABs, either with IMDM15 or with DMEM20 medium for human and murine adult cells respectively and MegaCell DMEM medium for hfMABs, at 37°C in a 5% CO_2/ 5% O_2 humidified incubator.
- Upon 100% confluence, remove medium, wash with PBS, add AD medium and incubate.
- Refresh medium every 2-3 days until appearance of adipocytes (approximately 10-14 days).
- Proceed to analyses (see Note 13 and Figure 3).

3.14 MAB chondrogenic induction

- Expand hfMABs with MegaCell DMEM medium at 37°C in a 5% CO_2/ 5% O_2 humidified incubator.
- Upon 100% confluence, remove medium, wash with PBS, add CD medium and incubate.
- Refresh medium every 2-3 days until appearance of adipocytes (approximately 10-14 days).
- Proceed to analyses (see Note 14 and Figure 3).

4. Notes

1. Murine adult MABs can be isolated from 7-day old mice until 4-week old onwards, however MAB proliferation and differentiation ability reduce with age.
2. Keep a thin layer of collagen coating onto the plates or dishes for 5 minutes at room temperature and before proceeding to culture cells, wash abundantly with PBS to remove any acid traces of collagen solution.
3. Let the dish bottom dry out to encourage tissue fragment adhesion. Then, incubate the fragment-containing 3.5 cm dishes in a sterile covered humid chamber containing a PBS lid-free dish, in order to avoid medium drop evaporation. Given that the isolation may take up to 10 days, check the PBS level regularly and eventually rinse it.
4. Erythrocytes start to sprout out in the first 48 hours and then after 72 hours fibroblast like cells containing hMABs should appear. hMABs look small, round-shaped and very bright cells. Once attached to the collagen layer, they start appearing as spindle-shaped cells.
5. In case of testing different Ab combinations, antibody quantities must be titrated according to manufacturer's datasheet.
6. If cells are plated directly into DMEM20 supplemented with extra antibiotics (0,5% gentamicin, 5% streptomycin, 5% penicillin), remove the medium 24 hours after plating, wash with PBS and rinse with 2 ml fresh DMEM20.
7. Murine adult MABs should be plated around 30-40% confluence. For instance, $< 5 \times 10^3$ cells should be plated in 1 well of 96 multi-well plates and between 5 to 10×10^5 in one well of 6 multi-well plates.

8. Human MABs generally need a longer incubation time (up to 14 days) to sprout out and be ready for sorting.
9. We recommend passing hMABS when 80% confluent at latest to prevent spontaneous cell fusions. Murine MABs are easily transduced to express fluorescent markers that can reveal C2C12 chimeric myotubes to assess their cell fusion potential. Myogenic differentiation potential should be confirmed by qRT-PCR, western blot and immunofluorescence analyses to detect late myogenic markers, such as myosin heavy chain or sarcomeric actin.
10. Human MABs have the ability to produce myotubes upon serum starvation, conversely to rodent MABs that need to be co-cultivated with muscle progenitor cells.
11. In smooth muscle cell induction, MABs should be kept at 60% confluence thus, it might be necessary to pass the cells during the differentiation process. Calponin and alpha smooth muscle actin should be detected by qRT-PCR, western blot, immunofluorescence and flow cytometry analyses, in order to quantify the smooth muscle differentiation rate.
12. Alizarin Red staining should be performed in order to reveal calcium deposits in MABs subjected to osteogenic induction, since proliferating MABs are already alkaline phosphatase positive.
13. Although we suggest Oil Red O for lipid-containing vacuole staining in MAB adipogenic induction protocol, Nile red staining or Peroxisome proliferator-activated receptor gamma detection can be also performed.
14. Safranin staining could be carried out for proteoglycans and glycosaminoglycans detection revealing chondrogenic differentiation of MABs as reported in Figure 3.

Acknowledgement

This work was supported by CARIPLO 2015_0634, FWO (G0D4517N), IUAP-VII/ 07 (EJJ-C4851-17/07-P), C1-KUL3DMUSYC (C14/17/111), Project Financing Stem Cells (PFO3 10/019), and Rondoufonds voor Duchenne Onderzoek (EQQFODUCH-O2010). MS is recipient of Hercules Foundation grant (AKUL/19/34) for the financing provided

to purchase the high throughput calcium imaging system.

References

1. S. Kuang, K. Kuroda, F. Le Grand, M. A. Rudnicki, Asymmetric self-renewal and commitment of satellite stem cells in muscle. *Cell* **129**, 999 (2007).
2. G. Cossu, S. Biressi, in *Seminars in cell & developmental biology*. (Elsevier, 2005), vol. 16, pp. 623-631.
3. M. Cassano *et al.*, Cellular mechanisms and local progenitor activation to regulate skeletal muscle mass. *J Muscle Res Cell Motil* **30**, 243 (Dec, 2009).
4. E. Agosti *et al.*, Both ghrelin deletion and unacylated ghrelin overexpression preserve muscles in aging mice. *Aging (Albany NY)* **12**, 13939 (Jul 26, 2020).
5. F. Ronzoni *et al.*, Met-Activating Genetically Improved Chimeric Factor-1 Promotes Angiogenesis and Hypertrophy in Adult Myogenesis. *Curr Pharm Biotechnol* **18**, 309 (2017).
6. A. Dellavalle *et al.*, Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. *Nature cell biology* **9**, 255 (2007).
7. A. Dellavalle *et al.*, Pericytes resident in postnatal skeletal muscle differentiate into muscle fibres and generate satellite cells. *Nature communications* **2**, 499 (2011).
8. M. Quattrocelli, D. Costamagna, G. Giacomazzi, J. Camps, M. Sampaolesi, Notch signaling regulates myogenic regenerative capacity of murine and human mesoangioblasts. *Cell death & disease* **5**, e1448 (2014).
9. D. Costamagna *et al.*, Smad1/5/8 are myogenic regulators of murine and human mesoangioblasts. *Journal of molecular cell biology* **8**, 73 (2016).
10. C. Bonfanti *et al.*, PW1/Peg3 expression regulates key properties that determine mesoangioblast stem cell competence. *Nature communications* **6**, (2015).
11. N. Breuls, G. Giacomazzi, M. Sampaolesi, (Epi)genetic Modifications in Myogenic Stem Cells: From Novel Insights to Therapeutic Perspectives. *Cells* **8**, (May 9, 2019).
12. E. Pozzo, Y. C. Chai, M. Sampaolesi, Comprehensive Overview of Non-coding RNAs in Cardiac Development. *Adv Exp Med Biol* **1229**, 197 (2020).
13. N. Giarratana *et al.*, MICAL2 is essential for myogenic lineage commitment. *Cell Death Dis* **11**, 654 (2020/08/18, 2020).
14. M. Sampaolesi *et al.*, Cell therapy of α-sarcoglycan null dystrophic mice through intra-arterial delivery of mesoangioblasts. *Science* **301**, 487 (2003).
15. M. Sampaolesi *et al.*, Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. *Nature* **444**, 574 (2006).
16. M. Quattrocelli *et al.*, Mesodermal iPSC–derived progenitor cells functionally regenerate cardiac and skeletal muscle. *The Journal of clinical investigation* **125**, 0 (2015).
17. G. Cossu *et al.*, Intra-arterial transplantation of HLA-matched donor mesoangioblasts in Duchenne muscular dystrophy. *EMBO molecular medicine*, e201505636 (2015).
18. M. Crisan *et al.*, A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell stem cell* **3**, 301 (2008).

19. F. L. Ronzoni, S. Lemeille, R. Kuzyakiv, M. Sampaolesi, M. E. Jaconi, Human fetal mesoangioblasts reveal tissue-dependent transcriptional signatures. *Stem Cells Transl Med* **9**, 575 (May, 2020).
20. F. R. Santoni de Sio *et al.*, Lentiviral vector gene transfer is limited by the proteasome at postentry steps in various types of stem cells. *Stem Cells* **26**, 2142 (Aug, 2008).
21. M. Quattrocelli *et al.*, Equine-Induced Pluripotent Stem Cells Retain Lineage Commitment Toward Myogenic and Chondrogenic Fates. *Stem Cell Reports* **6**, 55 (2016).
22. R. Tonlorenzi, A. Dellavalle, E. Schnapp, G. Cossu, M. Sampaolesi, Isolation and characterization of mesoangioblasts from mouse, dog, and human tissues. *Current protocols in stem cell biology*, 2B. 1.1 (2007).
23. A. Barbuti *et al.*, Mesoangioblasts from ventricular vessels can differentiate in vitro into cardiac myocytes with sinoatrial-like properties. *J Mol Cell Cardiol* **48**, 415 (Feb, 2010).
24. J. Camps *et al.*, Interstitial Cell Remodeling Promotes Aberrant Adipogenesis in Dystrophic Muscles. *Cell Rep* **31**, 107597 (May 5, 2020).
25. S. Picelli *et al.*, Full-length RNA-seq from single cells using Smart-seq2. *Nat Protoc* **9**, 171 (Jan, 2014).
26. A. Butler, P. Hoffman, P. Smibert, E. Papalexi, R. Satija, Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol* **36**, 411 (Jun, 2018).

Table1

Sample		***Positive*** ***(35-95%)***	***Negative*** ***(<5%)***
Murine skMABs		*SCA1+++, CD44+++, CD140b++, CD140a++, NG2+* single-cell RNA-seq analysis: Acta2, Anx5, Desmin, Pdgfr2	*CD45, CD31, Itga7, Ter119* single-cell RNA-seq analysis: Pdgfra, Fmod, Tnmd
Human skMABs			
	Adult	*AP+++, CD44+++, CD140b++, CD140a+, CD146+, CD13+++, CD90+, CD149+++*	*CD45, CD31, CD56*
	Fetal	*AP+++, CD146+, NG2++, CD90+++, CD13+++*	*CD45, CD31, CD56*

Table 1. ***MAB marker profile during proliferation evaluated by FACS and scRNA-seq***. + between 35% and 45% positive cells, ++ between 45% and 80% positive cells, +++ between 80% and 97% positive cells. Molecular MAB marker profile by scRNA-seq was directly performed on freshly isolated skeletal muscle cells.

Figure legends

Figure 1. scRNA-seq analysis of mononucleated lin$^-$ cells freshly isolated form murine muscles. (A) Schematic diagram showing the isolation of single cells from the hindlimbs of healthy mice with SMART-seq2. (B) UMAP plot and k-means clustering of cells from murine skeletal muscle identifying 6 clusters: Schwann cells, Smooth muscle cells (SMCs), Endothelial-like cells, Fibroblasts, Interstitial Stromal Cells (ISCs as reported in (*24*)) and activated Satellite Cells (MuSCs); each point represents one cell. (C) Heatmap of k-means clusters of differentially expressed marker genes.

Figure 2. Representative marker genes from single-cell analysis on fresh muscle. (A) Violin plot with median visualizing marker genes (Acta1, Myf5, Cd82, Acta2, Desmin, Pdgfr2 known as Pdgfrb, Anxa5 known as Annexin V, Vimentin, Pdgfra, Cd90, Fmod and Tnmd) for the identified clusters. All markers have been compared to literature (24).

Figure 3. Proliferation and differentiation of fetal MABs. A) Proliferating hfMABs show a star-shaped morphology comparable to human adult MABs. Scale bar = 100 mm. B) Growth curves showing hfMABs and hMABs population doublings (*$p<0.01$). C) Both hfMABs and hMABs are positive for AP staining. Scale bar = 10 mm. D) Representative images of hfMAB differentiation capacity are showed by IF analysis (Myogenesis: MyHC in red) and specific staining protocols (Adipogenesis: Oil Red O, Osteogenesis: Alizarin Red, Chondrogenesis: Safranin). Scale bar = 100 mm.

Figure 1

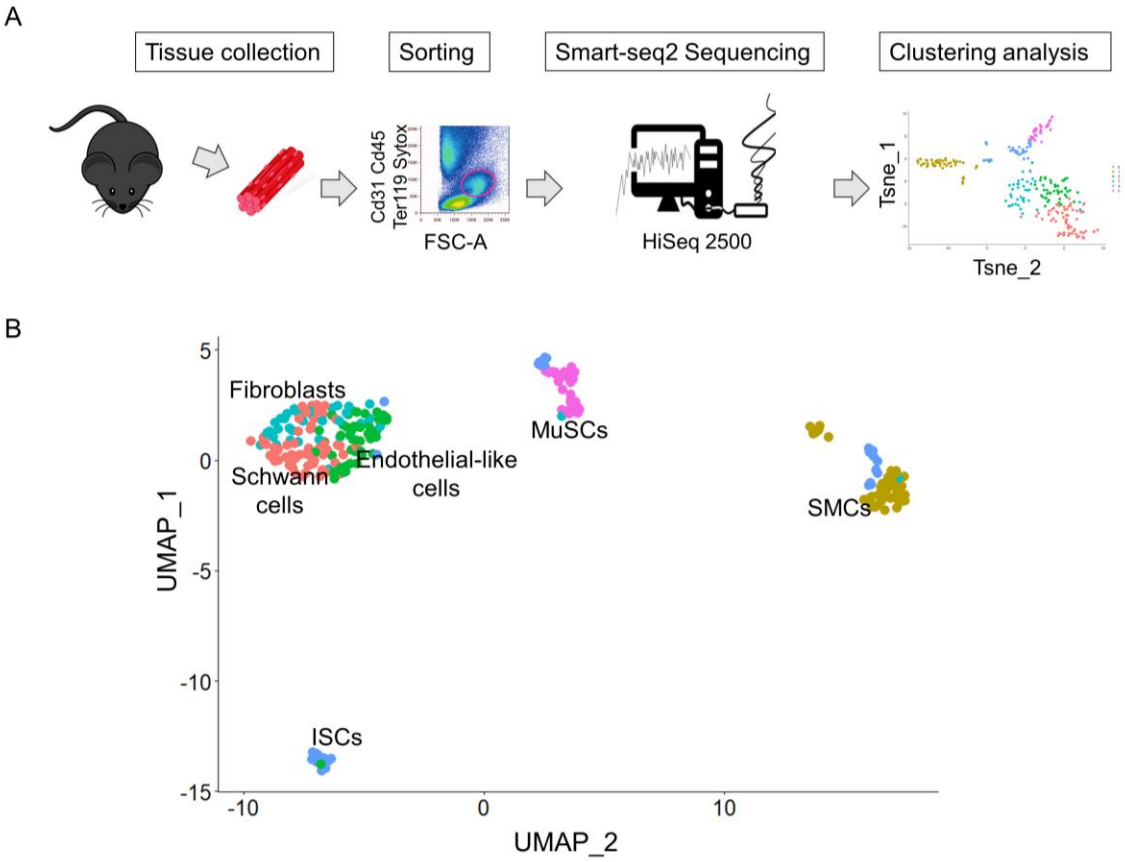

A
Tissue collection
Sorting
Smart-seq2 Sequencing
Clustering analysis
Cd31 Cd45
Ter119 Sytox
FSC-A
HiSeq 2500
Tsne_1
Tsne_2
B
Fibroblasts
MuSCs
Schwann cells
Endothelial-like cells
SMCs
ISCs
UMAP_1
UMAP_2
5
0
-5
-10
-15
-10
0
10
C
Schwann cells
SMCs
Endothelial-like cells
Fibroblasts
ISCs
MuSCs

Figure 2

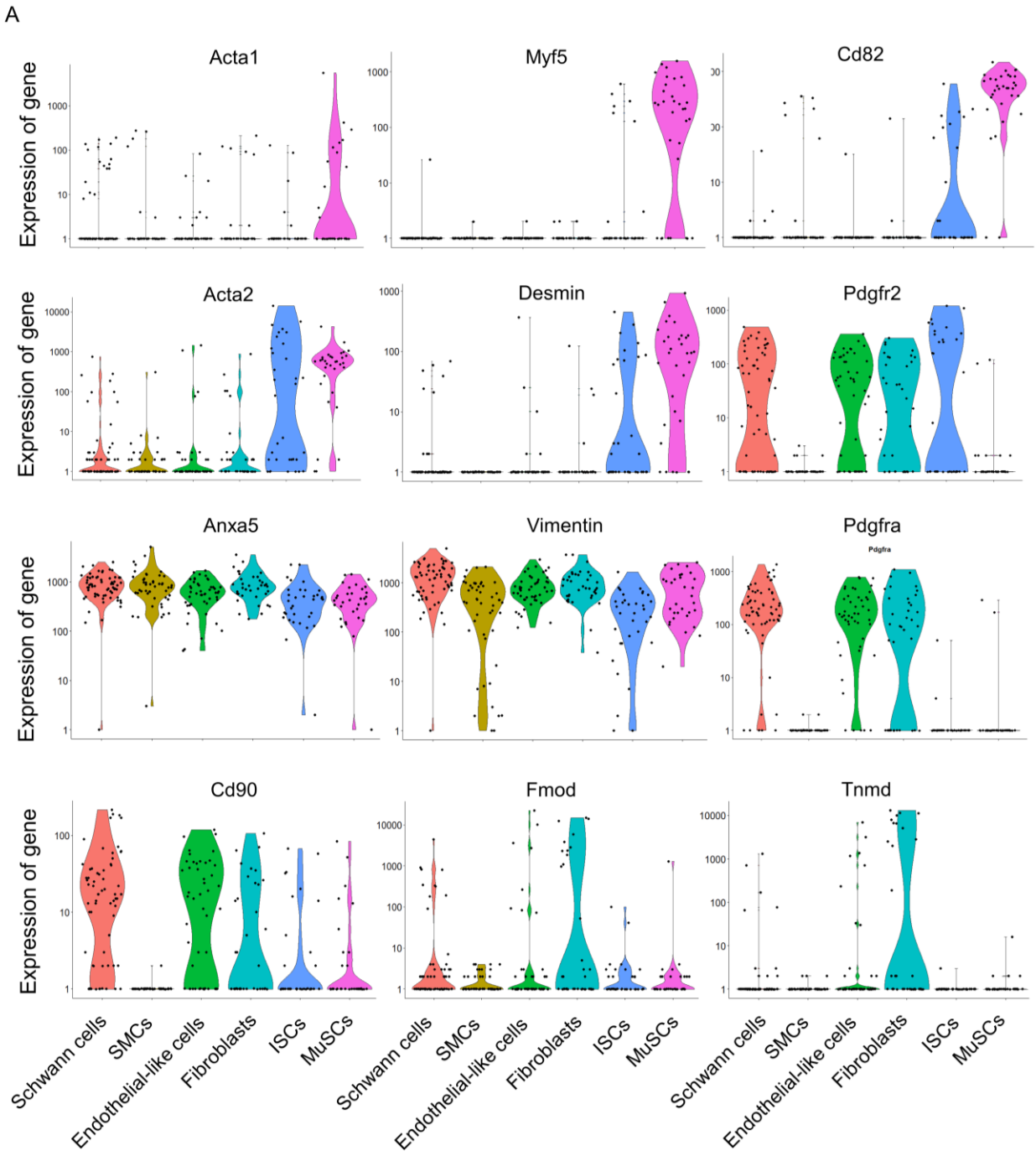
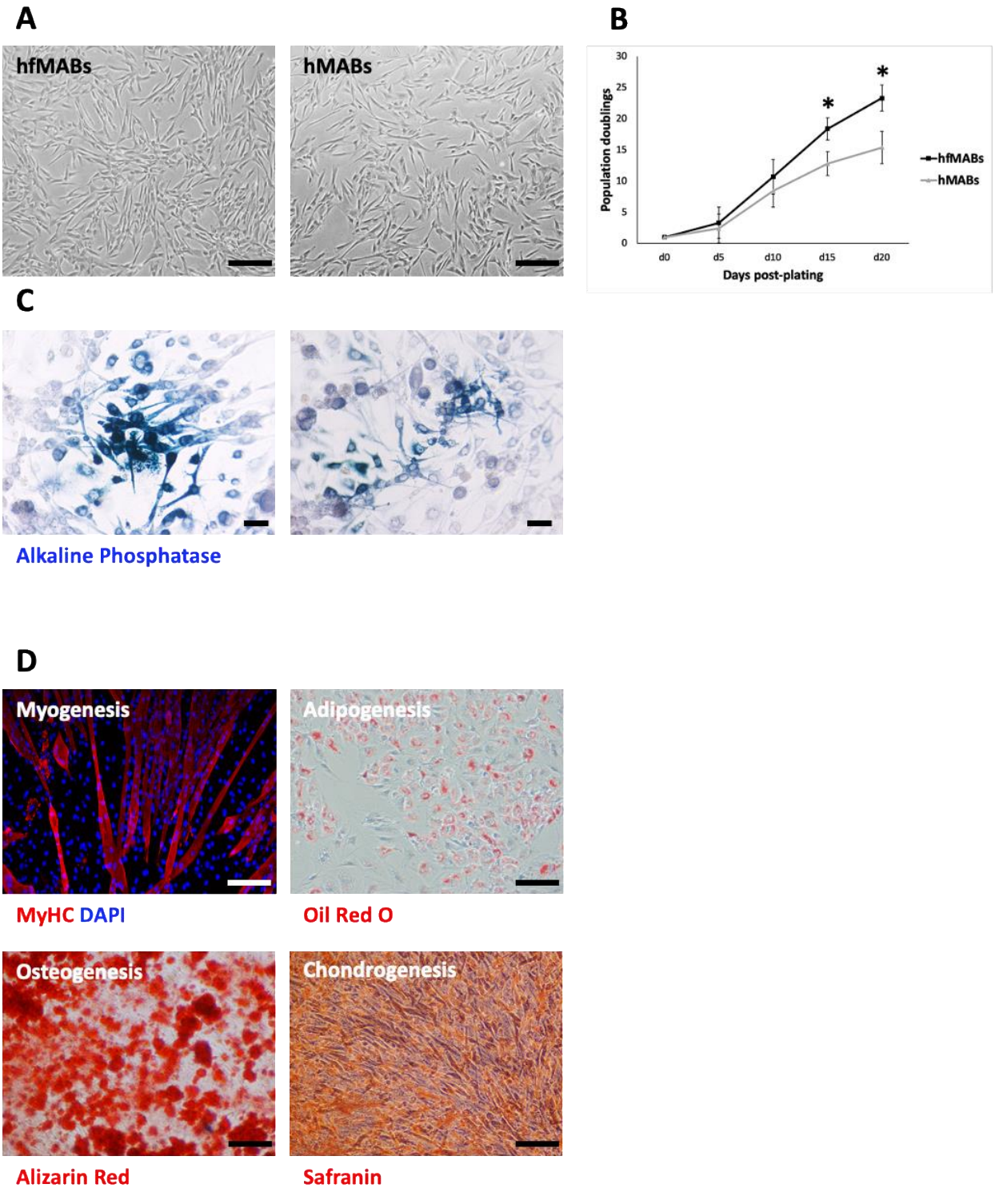

A
Acta1
Myf5
Cd82
Acta2
Desmin
Pdgfr2
Anxa5
Vimentin
Pdgfra
Cd90
Fmod
Tnmd
Expression of gene
Schwann cells
SMCs
Endothelial-like cells
Fibroblasts
ISCs
MuSCs

Figure 3


A
hfMABs
hMABs
B
Population doublings
Days post-plating
d0
d5
d10
d15
d20
hfMABs
hMABs
C
Alkaline Phosphatase
D
Myogenesis
Adipogenesis
MyHC DAPI
Oil Red O
Osteogenesis
Chondrogenesis
Alizarin Red
Safranin